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**Advances in hERG1 and LHR based targeting in
neoplastic and preneoplastic conditions**

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PREFACE

In this work we studied the role of hERG1 potassium channel and the luteinizing hormone receptor (LHR) in neoplastic and preneoplastic conditions. The present thesis is divided into three parts (three chapters). The first part concerns the depth study of hERG1 channel in Barrett's esophagus (BE) and its role during the progression to esophageal adenocarcinoma (EA), in order to (a) confirm hERG1 expression in a large cohort of BE patients (larger than the cohort studied in Lastraioli E et al 2006), (b) evaluate hERG1 expression during BE progression to esophageal dysplasia and EA. To deepen this topic and to better study the biology of hERG1 in BE, we developed three different mouse models of BE. Such models will be useful also to evaluate therapeutic interventions and pharmacological tests. This study was also aimed at testing the possibility of considering hERG1 as a marker of BE progression to be further exploited for BE surveillance screening protocols. The second part of the present thesis was aimed at studying the effects of LH over-expression induction in the endometrium *in vivo*. For this purpose we generated a transgenic mouse model over-expressing hLHR in the reproductive tract with the aim of determining whether: (a) LH/hCG-R over-expression is capable of inducing the development of Endometrial Cancer (EC) *per se*. (b) LHR over-expression in endometrial cells is might be responsible of a more aggressive behaviour of differentiated EC cells. This model could be a useful model not only for deciphering the genetic basis of EC development, but also for developing and testing novel therapeutic options in preclinical studies. In the last part of the thesis we evaluated the expression levels of LHR and two ion channels, such as hERG1 and KCNA7, in a cohorts of EC patients, in order to search for associations between the expression of the two genes and a number of co-factors including clinical and

pathological parameters. We have chosen to study LHR because it is proven to be involved in EC invasion and metastatic spread. As concerning hERG1 it was chosen because it was previously found to be aberrantly expressed in a several human tumors, as endometrial cancer, among others.

In addition, we also evaluated the mRNA levels of KCNA7 ion channel, supposed to be dysregulated in EC (Fortunato A., PhD thesis).

CHAPTER 1

1. INTRODUCTION

1.1 CLINICAL PRESENTATION OF BARRETT'S ESOPHAGUS (BE)

The esophagus serves as a dynamic tube, pushing food toward the stomach, where digestion and absorption take place. Mucus produced by the esophageal mucosa provides lubrication and eases the passage of food. Active peristaltic contractions propel residual material from the esophagus into the stomach. During reflux, the esophagus also serves as a passageway for gastrointestinal (GI) contents traveling retrograde from the stomach. The wall of the esophagus consists of four layers: mucosa, submucosa, muscularis propria, and adventitia (Kuo B et al 2006). The mucosa is composed by: a nonkeratinized squamous epithelium; a *lamina propria*, a thin layer of connective tissue; a *muscularis mucosa*, a thin layer of longitudinally, irregularly arranged smooth muscle fibers which separates the lamina propria from the submucosa. The submucosa contains connective tissue as well as lymphocytes, plasma cells, nerve cells, vascular network, and mucous glands. The esophageal glands are small racemose glands of mucous type and their secretion is important in esophageal clearance and tissue resistance to acid. The muscularis propria is responsible for motor function. The superior third is composed exclusively of voluntary striated muscle and the distal third is composed of smooth muscle. In between there is a mixture of both, called the transition zone. The adventitia is an external fibrous layer that covers the esophagus

composed of loose connective tissue and contains vessels, lymphatic channels, and nerve fibers (Kuo B et al 2006). Unlike other areas of the GI tract, the esophagus does not have a distinct serosal covering. This allows esophageal tumors to spread more easily and makes them harder to treat surgically. The missing serosal layer also makes luminal disruptions more challenging to repair. Originally the term “Barrett’s esophagus” was used to describe an esophagus in which a portion of the normal squamous mucosa was replaced by columnar epithelium. Since the 1950s, when the term became popularized, much has been learned about this condition, and new concepts continue to emerge that force reevaluation of this disorder. Gastroesophageal reflux disease (GERD) is a prevalent entity in Western countries and Barrett's esophagus (BE) is one of its main complications (De Meester S R et al 200). BE is a pathologic condition easily detectable at endoscopy and characterized by the replacement of the squamous epithelium of the distal esophagus by a columnar-type mucosa (intestinal metaplasia) (Figure 1).

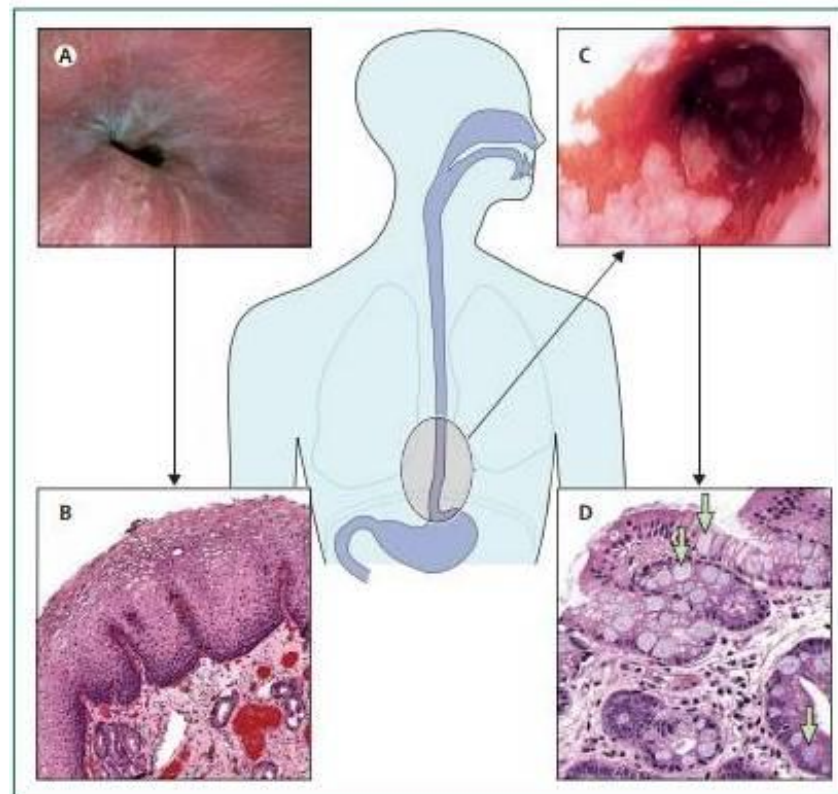


Figure 1: Normal esophagus versus Barrett's esophagus (A) Normal appearance of squamocolumnar junction at gastro-esophageal junction. (B) Normal esophageal squamous mucosa. (C) Tongues of Barrett's esophagus radiating orad from the gastro-esophageal junction. (D) Biopsy specimen of intestinal metaplasia (arrow points to goblet cell) (Shaheen NJ et al 2009).

In BE patients, examination by upper endoscopy show cephalad displacement of the squamocolumnar junction. The length of the displaced squamocolumnar junction should be measured during endoscopy: longer than 3 cm is long-segment Barrett's esophagus; 3 cm or shorter is short-segment Barrett's esophagus. Previously, investigators have suggested that short segments are not clinically significant, but other research has shown an increased cancer risk in even short-segment disease compared with the general population (Sharma P et al 1997; May A et al 2002). Therefore, the most common current definition of Barrett's oesophagus is salmon-coloured mucosa of any length in an oesophagus harbouring goblet cells. BE is precursor of esophageal

adenocarcinoma (EAC) and may progress to low-grade dysplasia (LGD), high-grade dysplasia (HGD) or adenocarcinoma (Shaheen NJ et al 2009).

1.2 EPIDEMIOLOGY

Barrett's esophagus is highly prevalent in the general population and especially in people with chronic reflux conditions, but in some patients the condition is asymptomatic. Decisions regarding endoscopic screening and understanding of the cancer risk partly depend on the prevalence of Barrett's esophagus in the general population. In a prospective study of a random patient sample, the prevalence of BE in the general population from Northern Europe who underwent upper endoscopy was 1.6%, very similar to the estimates made by experts, based on frequency of reflux symptoms and the presence of BE in patients with heartburn (Ronkainen J et al 2005). In studies of simultaneous endoscopy in healthy patients undergoing screening colonoscopy for colorectal cancer, the prevalence ranged from 5.6% in a US Midwestern population, 25 to 15–25% in elderly people (Rex DK et al 2003; Ward EM et al 2006). It follows that the real prevalence of BE in living adults is difficult to ascertain, because individuals with Barrett's are often asymptomatic and do not seek care. Moreover, the true prevalence depends also on demographic features (sex, age, ethnicity) together with geographical variations. In 1990, the prevalence in Olmsted County (USA), was about 21-fold higher than the clinically recognized cases in the county, suggesting that many, if not most, patients with Barrett's esophagus remained unrecognized. More than 40% of the patients with Barrett's esophagus reported no reflux disease and the investigators were unable to consistently identify risk factors for Barrett's esophagus in asymptomatic patients. Although the prevalence in the general population is substantial, it is much higher in patients undergoing upper endoscopy to investigate chronic reflux symptoms, at 5–15% (Westhoff B et al 2005). Zagari et al

analyzed 1033 patients originally identified as part of a large multicenter cross-sectional study on gallstone disease (Zagari RM et al, 2008). Of these patients, 1.3% (13) had histologically confirmed BE, while 0.2% (2) had long-segment BE. These estimates suggest that the prevalence of BE is between 0.5 – 2% of unselected individuals. In individuals with reflux symptoms, prevalence estimates are more variable, ranging from 5–15% (Westhoff B et al 2005; Runge TM et al 2015). The majority of the studies suggest an annual risk of adenocarcinoma to be 0.4–1% in patients with BE, representing a 50 to 70-fold increase compared with the general population (Alcedo J et al 2009). The incidence, prevalence, and risk of neoplastic changes in BE are not well established in the European Mediterranean area. Diet and lifestyle in this geographic area differ from those in other Western countries, and the prevalence of GERD is lower than reported for US population. Moreover, some European data (excluding data from the UK) suggest that the incidence of BE and the risk of adenocarcinoma development are lower than that observed in the USA (Alcedo J et al 2009).

1.3 RISK FACTORS FOR BE DEVELOPMENT

GERD: Gastroesophageal reflux disease (GERD) is a central risk factor for BE development. Numerous case-control studies have shown that individuals with GERD are 6–8 times more likely to have BE (Anderson LA et al 2007). Longer duration of GERD may create an environment conducive to the development of BE (de Jonge PJ et al 2014). However, the presence of reflux symptoms is neither sensitive nor specific for pathologic acid reflux, and indeed symptom severity does not correlate well with BE risk (Avidan B et al 2002). In any case, longer duration of GERD symptoms predicts increased likelihood of BE (Conio M et al 2002). In summary, reflux is associated with GERD, but symptoms of reflux cannot distinguish those with increased acid reflux from those without. It is likely that a genetic predisposing to the development of BE

combined with prolonged acid exposure and mutagenic events, including oxidative stress, may act synergistically in patients who develop EAC (Runge TM et al 2015).

Obesity: Obesity, measured by BMI and central adiposity, has been studied extensively as a risk factor for BE. The incidence of BE and EAC have risen dramatically in the past 40–50 years in Western societies, concurrent with rapid increases in the rate of obesity. From 1976 to 1991, the prevalence of obesity at all ages rose from 25% to 33%, and it now approaches 35% in adults (Ogden CL et al 2014). Obesity can be assessed in several ways. High body mass indices and especially central adiposity have been shown to have a significant association with BE. A 2009 meta-analysis that included 11 observational studies showed an increase in the risk of BE in patients with a BMI > 30 kg/m² compared to those with BMI < 30 kg/m² (Kamat P et al 2009). Patients with BE have been shown to have higher BMIs than either general controls or individuals with GERD but not BE (Spechler SJ et al 2011). Because BMI does not take into account the distribution of body fat, the estimated risk increase in the obese may actually be poorly estimated by BMI measurements. In fact, more recent work has shown that central adiposity, rather than BMI, may be the true driver of increased BE risk (Edelstein ZR et al 2007). It is possible that the male predominance among EAC cases could be explained in part by the fact that overweight males distribute fat preferentially to their trunk, and this central adiposity drives the risk increase (Berger NA et al 2013).

Alcohol: Alcohol use has been studied extensively as a possible risk factor for BE. Some work has suggested an inverse correlation between wine intake and BE risk (Thrift AP et al 2014). The most robust evidence we have on the issue comes out of the BEACON consortium, in which the data from 5 studies were pooled to assess risk of alcohol use (Kubo A et al 2009). Among 1,028 cases and 1,282 controls, alcohol use was stratified by gender and by number of drinks per day (Thrift AP et al 2014). There was a borderline significant inverse correlation between BE and any degree of alcohol

intake. Drinking 3 to <5 drinks per day was associated with a statistically significant reduction in BE risk, but with more or less alcohol consumed no statistically significant results were found. When assessing beverage-specific data, wine consumption was associated with an inverse risk of BE (Kubo A et al 2009). The preponderance of evidence supports no association between alcohol intake and BE risk. What was at one time thought to be a minor risk factor for BE now appears to confer no additional risk at all. Alcohol might indeed be protective, but in order to fully answer this question, more data are needed (Runge TM et al 2015).

Cigarette smoking: The majority of studies have found an association between cigarette smoking and an increased risk of developing BE (Cook MB et al 2014). However, there was significant heterogeneity between studies. To explore a possible synergistic effect between GERD and tobacco use in the genesis of BE, Cook et al. conducted modeling to assess the concurrent effects of smoking and GERD. They found that the Odds Ratio (OR) of Barrett's increased significantly when both GERD and smoking were present, compared to when smokers did not have GERD (Cook MB et al 2014). However, not all studies have identified smoking as a risk factor for BE. Thrift et al studied 258 patients with BE in a case-control study from Houston. These researchers found no association between smoking and BE in either group, even when stratified by pack-year exposures, length of time smoking, or number of cigarettes/day (Thrift AP et al 2014).

Helicobacter pylori: H. pylori infection can cause gastritis that decreases gastric acid secretion, protecting the esophagus from acid reflux and its complications (Fischbach LA et al 2012). H.pylori is also known to have a strong association with intestinal metaplasia in the body and antrum of the stomach (Stemmermann GN 1994). Temporal associations have been made between the decreasing prevalence of Hp infection in developed countries and the increasing prevalence of EAC. Corley et al showed that H.

pylori was inversely associated with BE in a case-control study design (el-Serag HB et al 1998; Corley DA et al 2008). While the mechanisms underlying this inverse association are not fully understood, they may relate to decreased acid production in the setting of *H. pylori* infection (especially with associated atrophic gastritis) or via alterations in the microbiome (Runge TM et al 2015).

1.4 PATHOGENESIS

The development of Barrett's esophagus is likely a two-step process. The first step involves the transformation of normal esophageal squamous mucosa to a simple columnar epithelium called cardiac mucosa. This occurs in response to chronic injury produced by repetitive episodes of gastroesophageal refluxing of acid and bile that damage esophageal squamous cells. The change from squamous to cardiac mucosa occurs relatively quickly, within a few years, while the second step, the development of goblet cells indicative of intestinal metaplasia, proceeds slowly, probably over 5-10 years (Oberg S et al 2012). Once present, Barrett's esophagus can progress to low- and high-grade dysplasia, and ultimately to adenocarcinoma. It is not known why the damage is repaired through columnar metaplasia rather than by regeneration of more squamous cells. Barrett metaplasia could result from transdifferentiation, in which squamous cells change into columnar cells through reflux-induced alterations in expression of key developmental transcription factors, or from transcommitment, in which esophageal stem cells (in the basal layer of the squamous epithelium or in the ducts of submucosal glands) that normally differentiate into squamous cells instead differentiate into columnar cells (Burke ZD et al 2012). Studies using mouse models has suggested that Barrett metaplasia might result from the proximal migration of stem cells from the gastric cardia or from expansion of a nest of residual embryonic cells at the gastroesophageal junction (Quante M et al 2012; Wang X et al 2011). The diagnosis of

Barrett's esophagus is suspected when an endoscopy reveals columnar mucosa in the esophagus and confirmed when biopsy specimens of that columnar mucosa show specialized intestinal metaplasia with its characteristic goblet cells. The distance between the gastroesophageal junction and the most proximal extent of Barrett metaplasia establishes whether there is long-segment (3 cm) or short-segment (<3cm) Barrett esophagus.

1.5 PROGRESSION OF BE TO EAC

The development of intestinal metaplasia within cardiac mucosa heralds the onset of a mucosal change that ultimately may lead to the development of esophageal adenocarcinoma. With continued inflammation and irritation of the metaplastic intestinal epithelium, some patients will progress through low-grade dysplasia, high-grade dysplasia, and subsequently adenocarcinoma. It is unknown whether the movement toward cancer is due to mitogenesis secondary to chronic mucosal injury, or mutagenesis as a consequence of exposure to a mutagen. One theory is that bile salts in their unionized state act as mutagens. If bile salts are demonstrated to contribute to the development of malignancy, then early intervention with an antireflux procedure should be encouraged (De Meester S R et al 2000). The precise risk of developing adenocarcinoma in patients with Barrett's esophagus is unknown, however, it likely is 0.2% to 2.1% per year for a patient without dysplasia, which is an incidence 30 to 125 times that of the general population (Provenzale D et al 1994). Although intestinal metaplasia is itself a premalignant condition, the development of high-grade dysplasia is associated with a significantly increased risk for adenocarcinoma. Several reports have correlated the progression of dysplasia within Barrett's with other cellular and genomic alterations, including mutations of the tumor suppressor gene p53, aneuploidy, and loss

of the Y chromosome (Krishnadath KK et al 1995; Krishnadath KK et al 1995) (Figure 2).

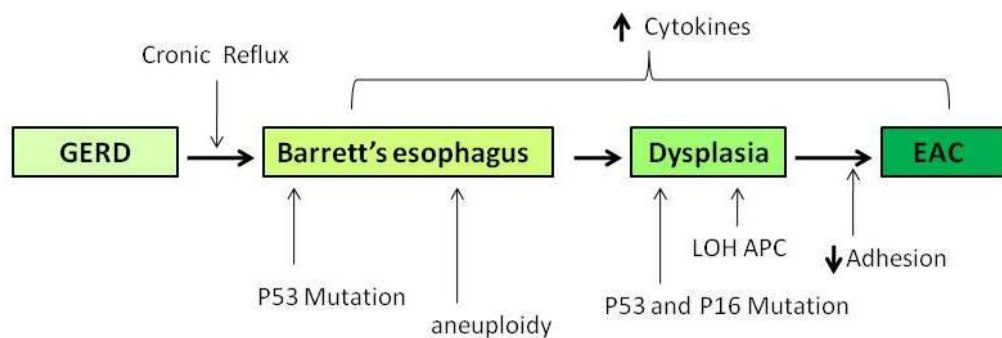


Figure 2: Description of the progression from GERD to EAC.

Importantly, any focus of intestinal metaplasia is capable of undergoing dysplastic change and ultimately becoming an invasive adenocarcinoma. Cameron and Carpenter have demonstrated that areas of dysplasia and cancer within long segments of intestinal metaplasia are often small and patchy, and that microscopic areas of different grades of dysplasia are often dispersed throughout the Barrett's. They suggested that dysplasia develops simultaneously in many areas and ultimately becomes confluent rather than spreading progressively outward from one site (Cameron AJ and Carpenter HA 1997). In addition, they noted that cancers developed throughout the length of the intestinal metaplasia, including distally near the stomach (Cameron AJ and Carpenter HA 1997). If intestinal metaplasia is present in a segment of columnar epithelium, it is always at the proximal end of the columnar segment. It may extend distally to involve most or all of the columnar segment, but it may be limited to the area near the squamocolumnar junction. Because available evidence suggests that adenocarcinoma occurs only within areas of intestinal metaplasia, it is likely that in these prior reports the intestinal

metaplasia was limited to the proximal portion of the columnar-lined distal esophagus (De Meester S R et al 2000).

1.6 MARKERS FOR PROGRESSION FROM BE TO DYSPLASIA/CANCER

Numerous studies have attempted to assess the utility of molecular biomarkers to predict progression and assist with risk stratification. If low risk patients can be accurately identified, then little or no follow-up may be warranted. Alternatively, chemopreventive or endoscopic interventions could be targeted to high-risk patients. However, to date none of the candidate biomarkers has been prospectively validated. P53 may have the most promise of any of the biomarkers for predicting neoplastic progression in patients with Barrett's. Kastelein et al studied the effect of aberrant p53 expression in a case-control study and found that P53 overexpression was associated with significantly increased risk of progression to either high-grade-dysplasia (HGD) or EAC (Kastelein F et al 2013). Among those with loss of p53 expression, the risk of progression was even higher. Low-grade-dysplasia (LGD) alone was far less predictive of progression and the positive predictive value of progression was 15% with LGD alone compared to 33% in patients with both LGD and aberrant p53 expression (Kastelein F et al 2013). Bird-Lieberman et al performed a retrospective, case-control study from a large cohort of patients in Northern Ireland. In that study, low-grade dysplasia, abnormal DNA ploidy, and *Aspergillus oryzae* lectin (AOL) were all risk factors for progression. LGD contributed significantly, but AOL and ploidy were also independent predictors of advancement to EAC (Bird-Lieberman EL et al 2012). A prospective study by Davelaar et al tested a protocol comparing p53 staining by immunohistochemistry and fluorescence in situ hybridization (FISH) to test on cytology specimens. They found that p53 abnormalities detected by immunohistochemistry and FISH were both independent predictors of progression (Davelaar AL et al 2015). In

addition, when both p53 and FISH were used, detection of LGD, HGD, and EAC was 100% accurate, both p53 and FISH improved the risk stratification capability of p53 alone.

Despite the significant advances in biomarker development, significant barriers remain. Of all the biomarkers currently identified, the greatest potential for clinical application may lie with assays using p53. P53 can be easily tested, and in multiple studies has been documented to improve the reproducibility of the diagnosis of dysplasia and to predict neoplastic progression (Timmer MR et al 2013). Due to the imperfect nature of dysplasia alone as a predictor of neoplastic progressions, work on molecular biomarkers continues. To date, however, no biomarkers are approved for diagnosis or risk stratification. Recent British Society of Gastroenterology guidelines propose that p53 immunostaining should be considered, in addition to routine clinical diagnosis, for BE diagnosis (Fitzgerald RC et al 2014). However, Pathology societies have yet to develop guidelines for the interpretation and reporting of p53 staining by IHC in Barrett's esophagus (Runge TM et al 2015).

1.7 ION CHANNELS IN CANCER

Ion channels are pore-forming transmembrane proteins that regulate passive ion fluxes that are important for key cell processes. They are good potential markers because of their localization at the plasma membrane level. Ion channels are progressively emerging as a novel class of membrane proteins aberrantly expressed in several types of human cancers. Besides regulating different aspect of cancer cell behavior, ion channels can now represent novel cancer biomarkers (Lastraioli E et al 2015). It is known that proliferating cells tend to be more depolarized than nonproliferating cells. As the cell cycle progresses, membrane potential fluctuates such that cells become relatively hyperpolarized just before the G1 to S transition, as K^+ channels allow K^+ to flow out of

the cell. Cells tend to depolarize as G2 transitions to M phase (Blackiston DJ et al. 2009, Boonstra J et al. 1981). Conduction through ion channels can regulate cellular proliferation in normal development of cells in mammals. For example, the expression of voltage-gated Kv1.3 (KCNA3) and Kv1.5 (KCNA5) subunits increases as cells progress from G0 to G1 in the cell cycle, causing a transitory increase in K^+ current during G1 in rodent glial progenitor cells. Inhibition of these channels arrest oligodendrocytes in G1 (Chittajallu R et al. 2002). Inhibition of delayed outward rectifying K^+ currents also can arrest glial precursor cells in G1, suggesting that the K^+ current regulates progression from G1 to S, at least in a normal developmental context in the brain (MacFarlane SN & Sontheimer H 2000). In the context of cancer, inhibition of voltage-gated K^+ channels slows the proliferation of cancer cells (Chang KW et al. 2003; Fraser SP et al. 2000; Menendez ST et al. 2010). Increased K^+ current promotes proliferation. In human breast carcinoma cells, increased expression of Kv1.1 (KCNA1) promotes proliferation, and inhibition of K^+ current slows proliferation (Ouadid-Ahidouch H et al. 2000). K^+ current, through KCNMA1, a KCa channel, also promotes the proliferation of breast cancer tumor cells, suggesting that the K^+ current rather than the specific type of channel is important for proliferation (Oeggerli M et al. 2012). Moreover, increased activity of voltage-gated K^+ channels promotes proliferation of colon cancer cells and malignant lymphocytes as well (Wang YF et al. 1992). Thus, in multiple cell types, different types of K^+ channels promote cell cycle progression from G1 to S, and inhibition of K^+ current can cause cell cycle arrest in G1. Recently, it was demonstrated that hyperpolarizing Cl^- channels promote cell cycle progression. For example, inhibition of volume-regulated Cl^- channels (VRCCs) causes p27 accumulation and G1 cell cycle arrest in human embryonic kidney (HEK) cells, small cell lung cancer cells, and T-cell leukemia cells (Renaudo A et al. 2007). Furthermore, inhibition of Cl^- intracellular channel-1 (CLIC1) in human glioblastoma cells induces

G1 arrest, suggesting that hyperpolarizing currents consisting of both K^+ and Cl^- are required for the G1 to S transition (Gritti M et al. 2014).

One hypothesis about how do mechanically ion channels effect proliferation is that ion channel activity activates a signaling cascade, eventually modifying molecular cell cycle proteins. A second hypothesis is that ion channels regulate cell volume and therefore the concentration of cell cycle regulatory proteins that directly affect cell cycle progression (Rouzaire-Dubois et al. 2000; Bates E 2014). Ion channels are involved also in cancer cells migration. Many Na^+ channel subunits are over-expressed in breast cancer cells, and increased expression correlates with increased metastasis (Chioni AM et al 2009). For example, the Nav1.5 α -subunit of a voltage-gated Na^+ channel is overexpressed in breast cancer cell lines that are highly metastatic, and phenytoin (an antagonist of this type of channel) inhibits metastatic properties (Yang M et al 2012). In general, data suggest that ion channels are involved not only in cancer proliferation but also in cell invasion and migration into neighboring tissue (Bates E 2014).

1.8 hERG1 CHANNEL

The *human ether-a-go-go related gene* (hERG) encodes the pore-forming subunit of a delayed rectifier voltage gated K^+ (VGK) channel. These channels are variously referred to as I_{Kr} , hERG, or Kv11.1 (Gutman GA et al 2003). Commonly, KCNH2 is used when referring to the gene and hERG when referring to the channel protein, also known as Kv11.1. Within the family of ether-a-go-go related, there are three members, Kv11.1 (hERG1), Kv11.2 (hERG2), and Kv11.3 (hERG3). KCNH2 was first cloned in 1994, by Warmke and Ganetzky, by screening a human hippocampal cDNA library with a mouse homolog of the *Drosophila* “*ether-à-go-go*” (EAG) K^+ channel gene (Warmke JW, Ganetzky B 1994). The main biological function of hERG1 channel is to determine the rapid component of the delayed rectifier potassium current (I_{Kr}) which

regulates the repolarisation of the cardiac action potential. Mutations in KCNH2 gene are the basis of chromosome 7-associated long QT syndrome (LQTS), an inherited disorder associated with a markedly increased risk of ventricular arrhythmias and sudden cardiac death (Curran ME et al 1995).

1.8.1 hERG1 Structure

KCNH2 gene is localized on chromosome 7, in q35-36 position, and the coding region comprises 16 exons spanning approximately 33 kb of genomic sequence. The major transcript of KCNH2 (hERG1a) contains 15 exons and the protein is composed of 1159 amino acids with a molecular mass of 127 kDa (Itoh T et al 1998;. Vandenberg JI et al 2012). hERG1 channel is formed by coassembly of four identical α -subunits arranged symmetrically around a central pore, with each subunit containing six α -helical transmembrane segments (S1-S6): S1-S4 segments constitute the voltage sensor domain (VSD) and S5-S6 along with the intervening pore loop (S5-P-loop-S6) contributing to form the K⁺-selective pore. Both amino- and carboxy-terminals are located in the cytoplasm (Warmke JW, Ganetzky B 1994) (Figure 3). The functional channel is a tetramer with the pore domain from each of the four subunits lining the central ion conduction pathway.

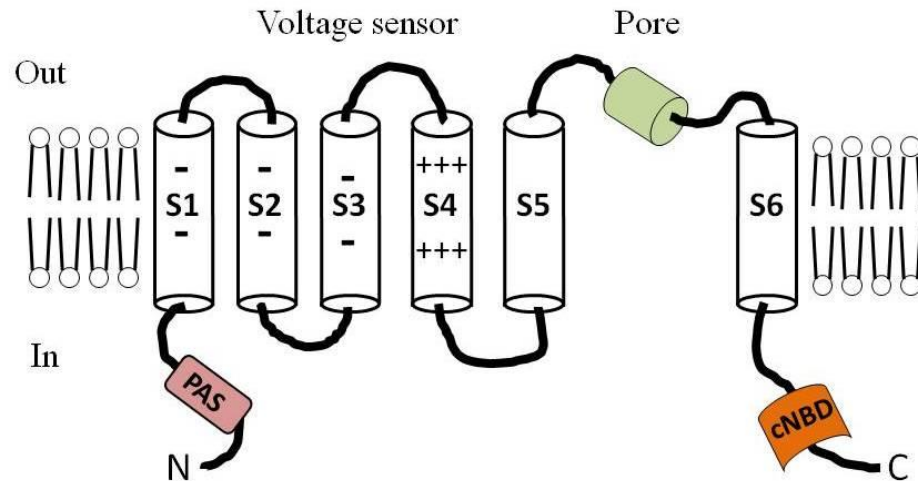


Figure 3: Structure of a single hERG containing six α -helical transmembrane domains: S1-S6. PAS: PAS (acronym of Per Arnt Sim) domain; cNBD (cyclic nucleotide binding domain) (Sanguinetti MC et al 2006).

Like other voltage-gated K^+ channels, hERG1 channel contains multiple positive charges in the S4 domain, because it is rich in basic amino acids (4 arginine and 2 lysines), and this acts as the primary voltage sensor for channel opening (Zhang M et al 2004). Mutagenesis of S4 identified Arginine 531 as the most important positively charged residue for proper voltage sensing in hERG (Subbiah, RN et al 2005). Positively charged amino acids in S4 domain and negatively charged acidic residues (Asp residues) in S1-S3 can form transient salt bridge during the gating (Sanguinetti MC et al 2006). S5-P-loop-S6 of each subunit contribute to shape the pore of the channel and to determine potassium selectivity. The extracellular end of the pore is a narrow cylinder called the K^+ -selectivity filter that is optimally constructed for conduction of K^+ ions (Sanguinetti MC et al 2006). The channel pore is asymmetrical and its dimensions change when the channel gates from a closed to an open state. Below the selectivity filter, the pore widens into a water-filled region, called the central cavity, that is lined by the S6 α -helices. In the large P loop there are 2 consensus site for N-glycosylation, which is important for the proper anchorage of hERG protein at the

plasma membrane (Gong Q et al 2002). In addition to the membrane-spanning region, the hERG protein contains large cytoplasmic NH₂-terminal and COOH-terminal domains. The NH₂ terminus contains a Per-Arnt-Sim (PAS) domain that defines the *ether-a-go-go* subfamily of VGK channels (Warmke JW, Ganetzky B 1994). The COOH terminus contains a cyclic nucleotide binding domain (cNBD), which shares homology with the cNBD of CNG channels and hyperpolarization activated channels (HCN). hERG1 channels can exist in closed, open, or inactivated states (Figure 4). In the closed state, at negative voltage, the four S6 domains criss-cross near the cytoplasmic interface to form a narrow aperture that is too small to permit entry of ions from the cytoplasm (Doyle DA et al 1998). In response to membrane depolarization, the S6 α -helices splay outwards and increase the diameter of the aperture to allow passage of ions. Membrane depolarization slowly activates the channels, which then inactivate rapidly, especially at higher potentials. The kinetic of inactivation of hERG1 channels is much more rapid than the kinetic of activation and the inactivation process is voltage-dependent (Vandenberg JI et al 2012).

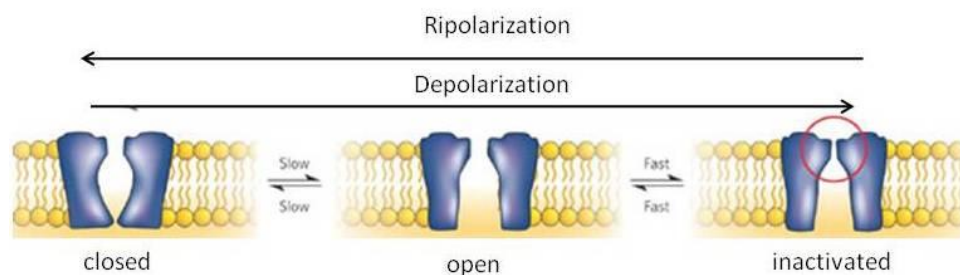


Figure 4: hERG channels are either closed, open or inactivated, depending on transmembrane voltage (Taken from MC Sanguinetti et al 2006).

1.9 ION CHANNELS AND ESOPHAGEAL ADENOCARCINOMA (EAC)

From a histopathological point of view, two types of cancer are the most frequent: squamous-cell carcinoma (ESCC) and adenocarcinoma (EAC). Barrett's Esophagus (BE) represents a precursor lesion for EAC. Although BE progression towards true

invasive cancer is not frequent, it represents a serious clinical problem, requesting frequent patients' endoscopic surveillance. Among voltage-gated K⁺ channels (VGKC), completely different patterns of expression were found between two members of the KCNH family: K_v10.1 (KCNH1) was expressed in ESCC compared with the corresponding normal tissue, the protein was associated with depth of invasion and was an independent negative prognostic factor (Ding XW et al 2008). On the contrary, K_v11.1 (KCNH2) potassium channels were shown to be expressed in precancerous lesions (BE, dysplasia) as well as in EAC (Lastraioli E et al 2006). It was also demonstrated that the K_v11.1 (KCNH2) channel is significantly associated with malignant progression towards EA. K_v11.1 (KCNH2) channels are also overexpressed in ESCC samples, but no statistically significant correlations emerged with clinicopathological characteristics. Nevertheless, K_v11.1 (KCNH2) expression negatively affects patients' survival (Ding XW et al 2008). Other channel types are expressed and functional in esophageal cancer cells. For example, TRPC6 is overexpressed in ESCC with respect to normal esophageal tissue at both protein and mRNA levels (Shi Y et al 2009). A recent report evidenced correlations of TRPC6 with T and staging and an association between TRPC6 mRNA and poor prognosis (Zhang SS et al 2013; Lastraioli E et al 2015).

1.10 hERG1 CHANNEL IN CANCER

In recent years, hERG1 channels has been detected in several types of human cancers and was found involved in many aspect of tumor progression: enhanced cell proliferation, angiogenesis, cell survival, invasiveness and metastasis (Bianchi L et al 1998, Arcangeli A et al 2009). hERG1 channels are overexpressed in human solid cancers of different histogenesis such as endometrial, colorectal, esophageal, pancreatic adenocarcinomas, as well as ovarian and brain cancers (Cherubini A et al 2000;

Lastraioli E et al 2004; Lastraioli E et al 2006; Feng J et al 2014; Asher V et al 2010; Masi A et al 2005). Data gathered in the last 15 years underlined that hERG1 channels are also important modulators of apoptosis and cell proliferation in leukemias and neuroblastomas (Wang H et al 2002; Pillozzi S et al 2002; Crociani O et al 2003). hERG1 channel can be considered a marker for malignant transition and its expression usually correlates to poor prognosis (Arcangeli A et al 2013; Lastraioli E et al 2012; Lastraioli E et al 2004). In this thesis we focused mainly on two types of solid cancer: esophageal cancer, which derives from Barrett's esophagus, and endometrial cancer.

hERG1 in esophageal cancer: As mentioned above, two types of esophageal cancer are the most frequent: esophageal squamous-cell carcinoma and esophageal adenocarcinoma. KCNH2 gene and hERG1 protein were found to be expressed in a high percentage of esophageal squamous-cell carcinoma samples but no correlations emerged with clinicopathological features. Survival rates of hERG1-positive patients were shorter than hERG1-negative patients (Ding XW et al 2008). In 2006, Lastraioli et al. showed that hERG1 is overexpressed in the majority of BE samples (69%) while it is absent in normal esophageal mucosa as well as samples taken from patients affected by esophagitis. It was also shown that hERG1 expression is switched on at early stages of BE cancerogenesis and it is also highly expressed in dysplasias and BE-derived adenocarcinomas, thus characterizing both early and late steps of esophageal cancerogenesis (Lastraioli E et al 2006). hERG1 channel expression also shows a significant association with malignant progression towards adenocarcinoma, since 89% of BE patients who developed EA were positive for hERG1 protein expression (Lastraioli E et al 2006).

2. MATERIALS AND METHODS

2.1 Patients and tissue specimens

Tissue samples were retrospectively obtained from different institutions belonging to GIRCG (Department of Clinical and Experimental Medicine, University of Florence; Pathology Division, Azienda Ospedaliero-Universitaria Senese; Pathology Division, Borgo Trento Hospital, Verona; Pathology Division, Morgagni-Pierantoni Hospital, Forlì; Pathology Division, Esine Hospital, ASL Vallecamonica Sebino; Institute of Pathology, Spedali Civili, Brescia). A total of 125 BE, 16 ED and 25 EA paraffin-embedded samples were collected.

Diagnosis and histological grading were assessed in all cases using standard criteria by experienced pathologists (LM, CV, AT, LS, MC and VV).

2.2 Immunohistochemistry

Immunohistochemistry was performed as previously reported using an anti-hERG1 monoclonal antibody directed against the S5-pore region (Dival Toscana Srl) at 1:200 dilution and slides were incubated overnight at 4°C. Immunostaining was performed with a commercially available kit (PicTure Max kit and DAB, Invitrogen).

2.3 Scoring assessment

Samples were evaluated applying a scoring system frequently used for cytoplasmic and membrane proteins. Such scoring system combines the estimate of the percentage of positive cells with the staining intensity. Staining intensity was rated on a scale of 0–3, with 0 = negative; 1 = weak; 2 = moderate, and 3 = strong. The raw data were converted

to the combined score by multiplying the percentage and staining intensity values, obtaining a value between 0 and 300 for each sample. Only samples with a complete score equal to 0 were considered negative.

2.4 Retrospective Case-Control Study

A case-control study design has been chosen because the study addresses the potential association between a rare medical condition (EA in BE) and a relatively common marker (hERG1 expression). Cases are defined as BE subjects whose lesions progressed towards dysplasia/adenocarcinoma while Controls are BE patients, with at least a follow-up visit completed during the last 10 years, whose lesions didn't progressed at the last time when they were examined. Since there may be a considerable lag time between the diagnosis of BE and the progression towards EA, the date of BE diagnosis is defined as index date. Three controls will be individually matched per case for age at the index date and gender. Controls will be randomly chosen, without replacement, between all individuals with a follow-up duration equal to or longer than the interval between index date and EA diagnosis in the corresponding case.

The number of samples collected reached a case-control ratio equal to 1:3 (26 progressed BE and 78 not-progressed BE). The study population was therefore represented by 104 patients with complete follow up information. We calculated that, for a two-sided alpha error equal to 5%, with 15 cases available, a case-control ratio equal to 1:3, and a prevalence of hERG1 expression between controls equal to 10%, the study will have a power of 80% against a minimal detectable risk (odds ratio) of 6.7. According to our previous data, such a strong association should be plausible.

2.5 Statistical analysis

Standard univariate and multivariate logistic regression analyses were performed to assure that the risk estimates for progression towards EA are appropriately adjusted for confounding and effect modification.

Data were collected locally and thereafter transferred to the Clinical Trials Coordinating Centre (CTCC) of the Istituto Toscano Tumori, according to clear time lines and defined responsibilities. Incoming data will be checked in the CTCC for quality and comprehensiveness by a quality assurance system and queries will be done to the study center if there is as doubt about the validity of the data or if there are missing data. Statistical analysis were performed in collaboration of Dott. L.Boni (University of Florence).

2.6 BE induction in mice

13 Balb-C, 39 CD-1 mice (2-4 months of age, weighing 24-29g) and 33 FVB hERG1 transgenic mice (2-4 months of age, weighing 23-30g), overexpressing hERG1 in GI tract [13], were operated to perform gastro-jejunal anastomosis (EJA), inducing gastro duodenal mixed reflux. Anesthesia was performed by an intraperitoneal injection of Avertin 2.5% (16 μ g/g body weight). A total of 85 mice were operated and only 15 of them survived after surgery: 6 CD-1, 5 Balb-C and 4 FVB hERG1 TG mice. We hypothesized that the high early mortality might be related both to hypothermia (caused by the heat loss from the abdomen) and anesthesia.

Surgery was performed by placing the mouse on a small surgical table and, after a 2-3 cm median incision of the skin and peritoneum, the esophagogastric junction was exposed. Distal esophagus was sectioned after clamping in order to prevent esophageal retraction and the gastric side stitched, at cardiac level, with suture thread Goretex 8/0. A small hole was performed in the jejunum wall by a 14 Fr intravenous cannula and

then the esophagus-jejunal anastomosis with silk 7/0 was performed. After successful repositioning of the viscera the abdominal wall was closed with nylon 4/0. A representative picture showing the result of the surgical procedure is reported in Fig 2A. Mice were sacrificed after 9 and 12 months. Furthermore, another set of experiment was performed using a mixed chemical-transgenic model of BE treating 10 3-month-old IL-1 β TG mice with 0.2% deoxycholic acid (DCA) in the drinking water (pH 7.0). 7-8 months after the beginning of the DCA-treatment mice were sacrificed.

2.7 Histological and immunohistochemical analysis

After the animal sacrifice the stomach and esophagus were removed and fixed in 4% formalin for 24 hours. Thereafter, samples were processed for paraffin embedding and 7 μ m longitudinal cut sections were obtained through a microtome and put on positive-charged slides.

Samples were stained with Hematoxylin/Eosin and Alcian Blue standardized protocols to detect goblet cells and then observed under a light microscope.

Moreover, samples were stained with anti-hERG1 polyclonal antibody, in order to evaluate the expression of hERG1 channel. The antibody was diluted in UltraVBlock (LabVision) in PBS1:10 (v/v) at a final dilution 1:200.

2.8 TG mouse models

In this work we used two different transgenic mouse models. The first one was hERG1-transgenic (TG) mouse model, which over-express hERG1 in the mucosa of the large intestine. This model was developed by our laboratory and was described in Fiore A 2008 (Fiore A. et al 2013). Briefly, the hERG1 cDNA, tagged with the myc epitope and a poly-histidine (His) flag at the protein C-terminal, was put under the control of the human b-actin minimal promoter, with an intercalated floxed reporter EGFP gene,

which should block transgene transcription. The floxed EGFP gene was used to conditionally express hERG1 in gastro-intestinal tract. Unfortunately, because of transcriptional readthrough phenomenon TG mice expressed hERG1 both in the colon and in the liver even in absence of Cre-mediated recombination. Therefore, mice, due to the transcriptional control exerted by the b-actin promoter, can be considered to over-express the hERG1 transcript ubiquitously.

The second model was IL-1 β transgenic (TG) mouse model. IL-1 β transgenic mice were generated by targeting expression of hIL-1 β to the esophagus using the Epstein Barr virus promoter (EB-L2) that targets the oral cavity, esophagus and squamous forestomach. This model was widely described in Quante M 2008 (Quante M et al 2008).

3. AIMS OF THE STUDY

The present study was designed with the aims of: investigating hERG1 channel expression in a large cohort of BE patients using a monoclonal antibody anti-hERG1; determining the expression of hERG1 during BE progression toward esophageal adenocarcinoma. In this study we tested also the possibility of considering hERG1 as a progression marker in BE. In order to better study the biology of BE at the onset of the disease we developed three different BE mouse models: (a) a surgical model, obtained by esophagojejunal anastomosis (EJA); (b) a mixed chemical-transgenic model, treating IL-1 β TG mice with deoxycholic acid; (c) a mixed surgical-transgenic model, in which EJA was applied in a hERG1 TG mouse model.

1. RESULTS

4.1 hERG1 expression in BE and during esophageal tumor progression.

We previously showed that hERG1 expression was up-regulated in BE samples, compared to both normal esophageal mucosa and GERD samples without or with esophagitis (Lastraioli E et al 2006). In the present work we validated our previous data in a larger cohort of BE samples (125 patients), using an anti-hERG1 monoclonal antibody (Mab-hERG1, Dival Toscana Srl; Sesto Fiorentino, Italy) instead of the anti hERG1 polyclonal antibody previously used. The Mab-hERG1 was previously proven to give a clearer signal and hence easiness of interpretation. This antibody recognizes an extra-cellular epitope of hERG1 protein and for this reason can be used without permeabilization. No hERG1 expression was detected either in normal squamous epithelium (Figure 1A) or in areas displaying signs of esophagitis (Figure 1B). Instead, the expression of hERG1 can be clearly observed in metaplastic cells composing BE tissue (Figure 1C), but not all BE samples are hERG1 positive (Figure 1 D).

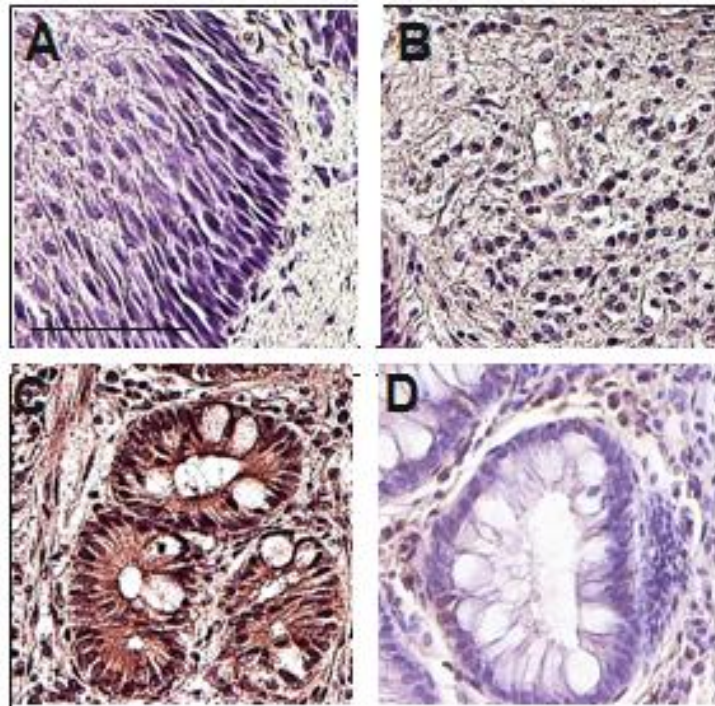


Figure 1: hERG1 expression in human esophageal carcinogenesis. IHC was performed as described in Materials and Methods with anti-hERG1 monoclonal antibody. **A)** Normal esophagus. **B)** Esophagitis. **C)** Representative BE sample expressing the hERG1 protein. **D)** Representative BE sample negative for hERG1 expression. Original magnification 20x. Scale bar: 100nm.

These results not only confirm what previously published by Lastraioli et al (Lastraioli E et al 2006), but also show the lower background, with easiness of interpretation of the Mab-hERG1 labelling. Overall, hERG1 was expressed in 48% (60/125) BE samples (see also the bar graph in Figure 2A). We then defined a scoring system, taking into account both the percentage of labelled cells and the staining intensity. The score was obtained by multiplying the two values and was therefore ranging from 0 (negative) to 300. Considering only positive samples (with scores >1), it emerged that BE samples have a median score equal to 145 ± 86.03 (n=60).

We also have collected and analyzed sixteen ED and twenty-five EA cases and hERG1 expression was evaluated. Overall, hERG1 was expressed in 14 out of 16 (87,5%) ED and in 24 out of 25 (96%) EA (Figure 2A). When the positive samples were scored

according to the scoring system described above, BE samples showed a significantly lower median score (145 ± 86.0), compared to ED (255 ± 70.7 , BE vs ED $p=0.023$) and EA (270 ± 48.4 , BE vs EA $p<0.001$) (Figure 2B).

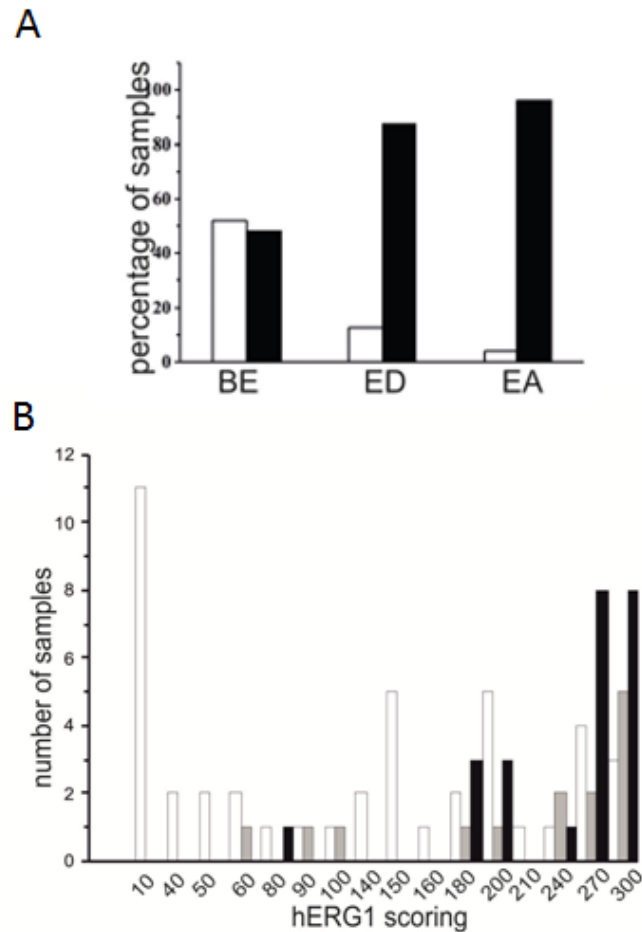


Figure 2: **A)** Histogram summarizing hERG1 expression in the three different groups of samples analyzed (BE, ED and EA). White bars: hERG1 negative samples, Black bars: hERG1-positive samples. **B)** Histogram summarizing hERG1 scoring in the different groups (BE, ED and EA). Samples were scored as described in Materials and Methods. Analysis performed using 2-sided Student's T test revealed statistically significant differences between BE and ED ($p=0.013$), between BE and EA ($p<0.001$) and between ED and EA ($p=0.007$). White bars: BE; Grey bars: ED; Black bars: EA.

Five cases, for which the entire progression from BE to ED and then EA was available, were fully analyzed and scored. Representative samples of BE, ED and EA are shown in Figure 3A-C.

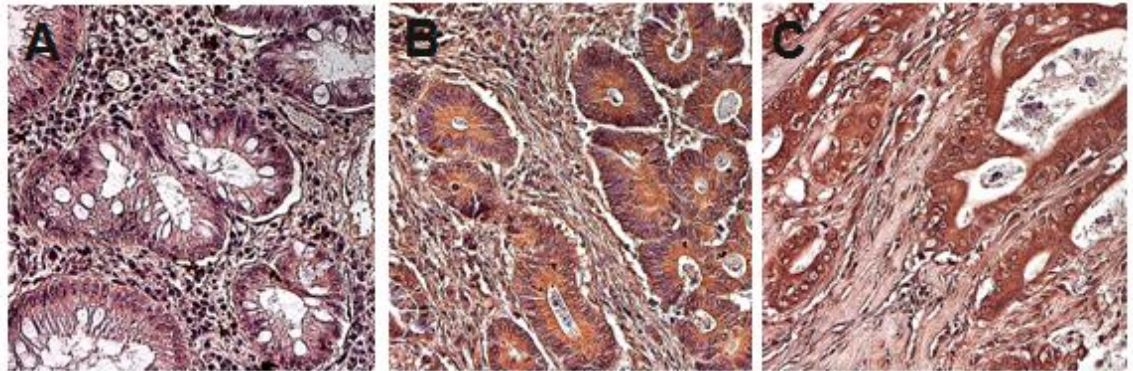


Figure 3: hERG1 expression increase during EC progression to EA. **A)** BE sample. **B)** Esophageal Dysplasia (ED). **C)** Esophageal Adenocarcinoma (EA). Original magnification 20x.

We evaluated hERG1 expression in BE, ED and EA of all the five cases and we analyzed the scorings: hERG1 positive BE samples showed a trend to increase hERG1 expression during progression (Figure 4).

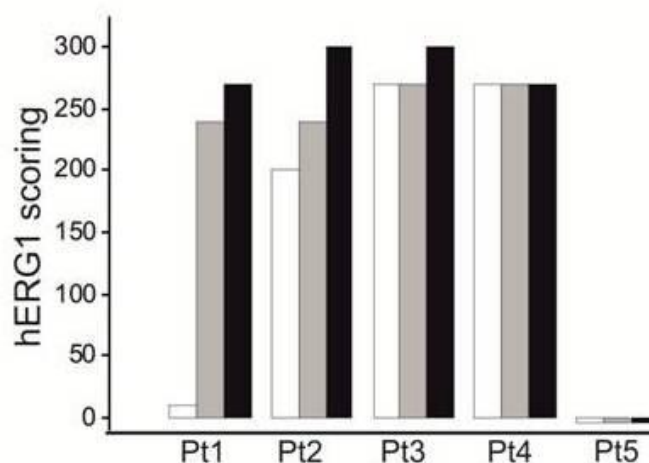


Figure 4: Histogram summarizing hERG1 scoring in the different samples (BE, ED and EA) of 5 representative patients. White bars: BE; Grey bars: ED; Black bars: EA.

Collectively, these data allowed us to conclude that hERG1 is expressed in approximately half of BE samples, and its expression increases during BE progression to adenocarcinoma.

4.1 Case-control study.

Subsequently, we investigated whether hERG1 might represent a biomarker of tumor progression in BE. To this purpose we tested whether hERG1 was differentially expressed in progressed and not-progressed BE sample. We analyzed 104 BE primary samples with a follow-up of at least 10 years, provided from different institutions in Italy (GIRCG network). Accordingly, samples were divided in two groups: samples that progressed towards ED and/or EA in the follow-up time (pBE) and samples belonging to patients whose lesions did not progress to EA (npBE). The ratio between cases (pBE) and controls (npBE) was 1:3. For the case-control study samples were scored only as “positive” and “negative” for hERG1 expression. Representative examples of a npBE sample, which does not express the hERG1 protein, and of a pBE sample, which is positive for hERG1 expression are shown in figure 5A and 5B, respectively.

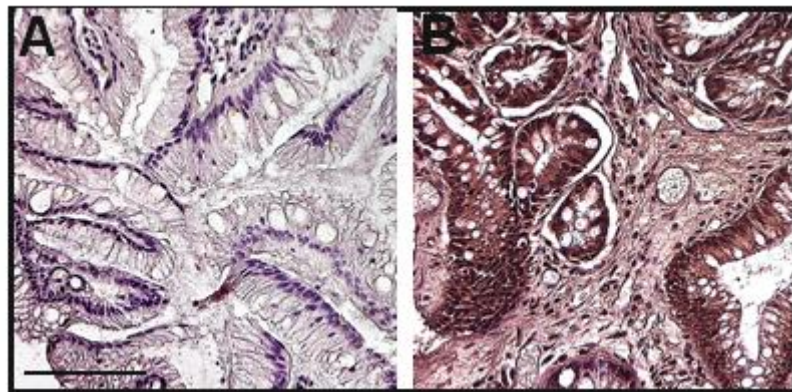


Figure 5: hERG1 expression along tumor progression. **A)** Non-progressed BE. The representative sample here reported is negative for hERG1 expression. **B)** Progressed BE. A representative hERG1-positive sample is shown. Original magnification 20x. Scale bar: 100nm.

Overall, hERG1 channel was expressed in 73,1% of pBE (19 out of 26) while only 42.3% (33 out of 78) npBE expressed hERG1 (Figure 6).

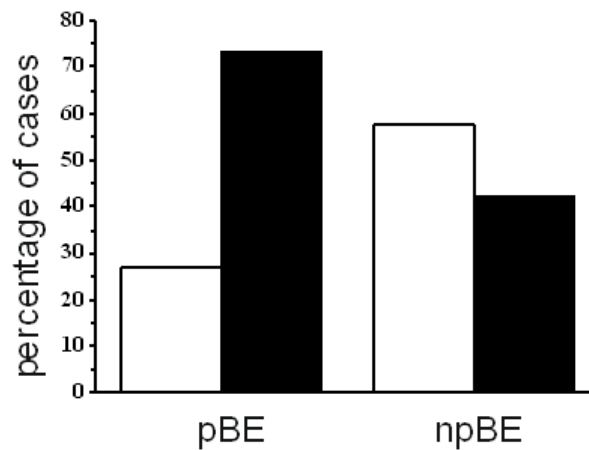


Figure 6: Histogram showing hERG1 expression in progressed (pBE) and non-progressed BE (npBE). White bars: hERG1 negative; Black bars: hERG1 positive.

Statistical analysis indicated a statistically significant association between hERG1 expression in BE and the risk of progression to EA (Odds ratio= 3.70, 95% CI (Confidence Interval): 1.40-9.82; P=0.006) (Table 1).

	npBE (%)	pBE (%)	Odds Ratio	95% CI	P value (likelihood ratio test)
hERG-	45 (57,7%)	7 (26,9%)	3,70	1,40-9,82	P=0,006
hERG+	33 (42,3%)	19 (73,1%)			

Table 1- Statistical analysis for the case-control retrospective study. npBE: not progressed BE, pBE: progressed BE.

4.3 hERG1 expression in BE lesions of BE mouse models

To better investigate the role of hERG1 channel in BE progression to EA, we also developed different BE mouse models, in which BE lesions were induced either surgically or chemically. The surgical model consisted in mice in which an esophago-jejunal anastomosis (EJA) was performed (Figure 7A,B,C). For this purpose we used

either CD1 or Balb-C mice. Mice were sacrificed 9 to 12 months after surgery, and esophagus, stomach (atrophic) and intestine were removed to be analyzed for the detection of BE lesions through hematoxylin and eosin as well as Alcian blue staining (Figure 7D). All the operated animals showed the signs of gastric mucosal atrophy due to the EJA (Figure 7B,C). 4 out of 11 (36%) operated animals developed histologically detectable intestinal metaplasia in the lower esophagus, indicative of BE (Figure 7E, red arrow). One mouse also showed signs of ED in an area surrounding a BE lesion (Figure 7E, yellow arrow).

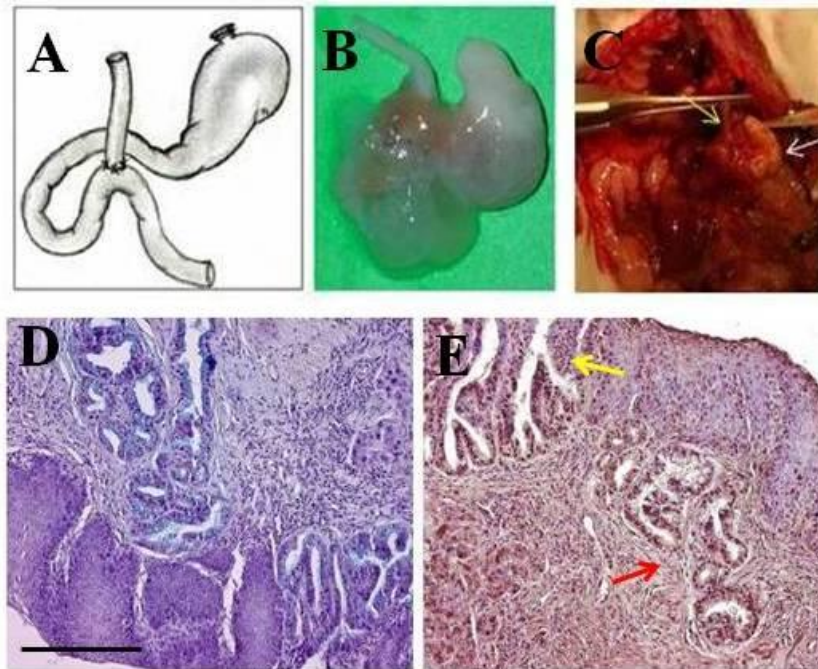


Figure 7: A) Drawing of the EJA performed in this study; B) representative specimen from an operated animal, showing EJA; C) EJA at the moment of the sacrifice: the yellow arrow indicates the esophagus, the white one indicates the atrophic stomach. D) Alcian Blue staining performed on Balb-C mice in order to detect goblet cells; E) IHC performed on Balb-C mice, in order to evaluate hERG1 expression, indicates that hERG1 is not expressed in normal esophageal tissue while it is up-regulated in metaplasia (red arrow) and dysplasia (yellow arrow). Original magnification 20x.

The second was a model of chronic esophageal inflammation: IL-1 β transgenic mice received, in drinking water, 0.2% deoxycholic acid (DCA) for 7 months of treatment (as described in Materials and Methods) (Figure 8A). For this reason this model was a mixed “chemical-transgenic” model. Four out of 10 (40%) IL-1 β TG DCA-treated mice developed histologically detectable BE lesions (Figure 8B).

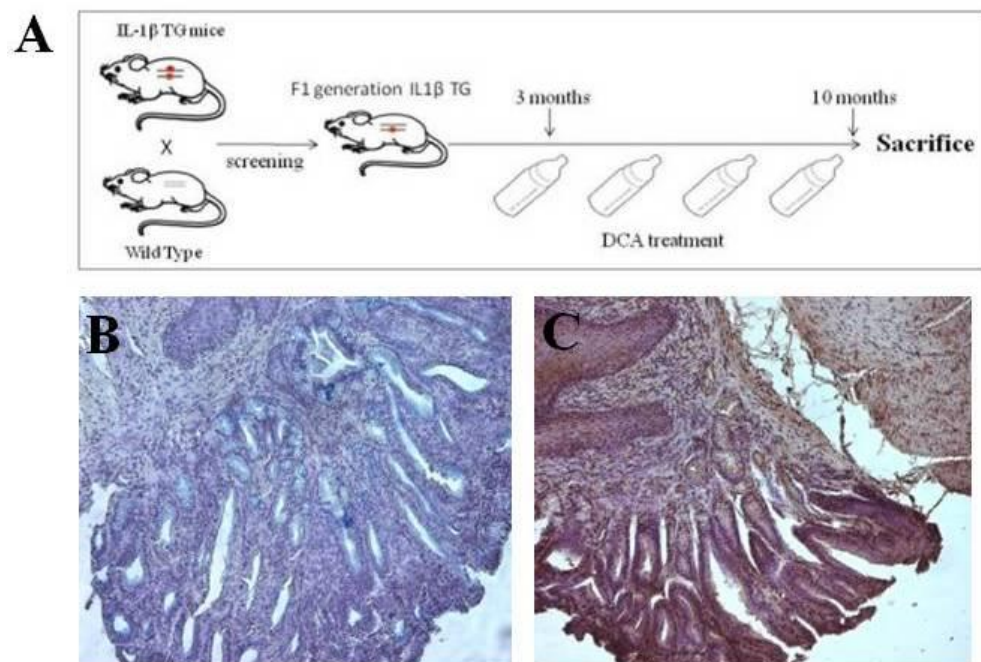


Figure 8: A) From left to right: birth and screening of IL-1 β TG mice F1 generations (chemical-transgenic model of BE): each founder mouse is bred with a Wild Type partner. The IL- β TG mice are treated with 0,2% DCA for 7 months and then sacrificed. Alcian Blue (B) and IHC (C) staining performed on IL-1 β TG mice DCA-treated in order to detect BE lesions and to evaluate hERG1 expression. Original magnification 20x.

An IHC analysis was performed to evaluate the expression of hERG1 in BE lesions of either mouse models. It emerged that hERG1 was absent in normal tissue and in esophagitis in both types of models, while it is up-regulated in BE lesions of both mouse models (Figure 7E, Figure 8C, and Figure 9A) (mean score=130 \pm 28.7 in the surgical model and mean score = 113 \pm 37 in the chemical-transgenic model) as well as in the dysplasia observed only in the surgical model (mean score=270) (Figure 9B).

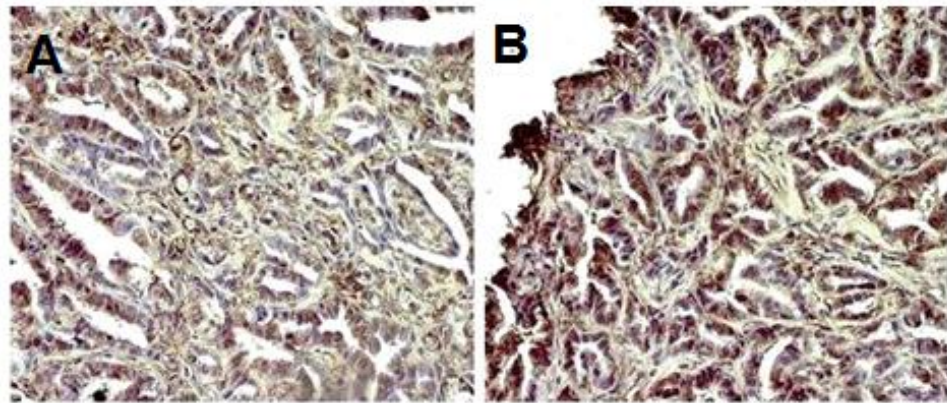


Figure 9: Detail of metaplastic (A) area and dysplastic area (B) of a Balb-C mouse sample. Original magnifications, 40x.

Finally, we investigated whether BE lesions induced by the EJA surgical procedure occurred at higher frequency in mice over-expressing hERG1, we operated FVB hERG1 transgenic (TG) mice whose hERG1 over-expression in the GI tract was proven in Fiore A. 2013 (Fiore A. et al 2013). This model was a mixed “surgical-transgenic” model. Alcian Blue staining was also performed to detect intestinal metaplasia (Fig 10A), as above. Four out of 4 (100%) FVB hERG1 TG mice developed BE lesions 9-12 months after surgery (Fig 10B).

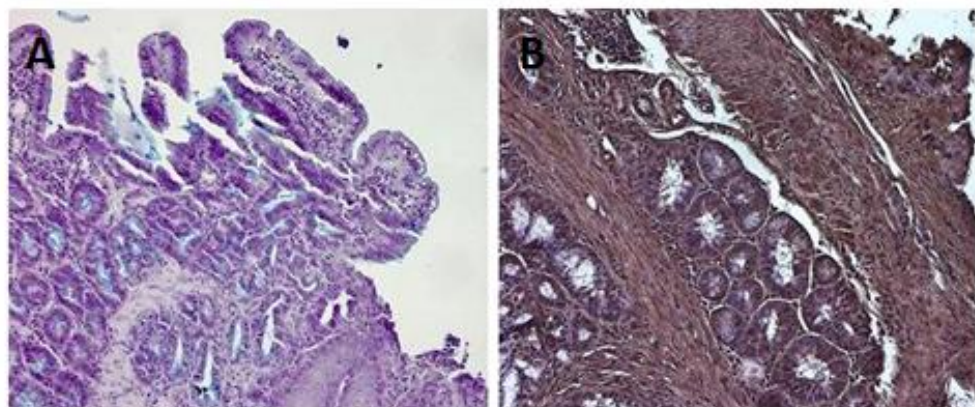


Figure 10: A) Alcian Blue staining shows BE lesion in hERG1 TG mice ; (B) IHC performed on hERG1 TG mice confirm the HERG1-positive labelling. Original magnifications, 20x.

5. DISCUSSION

BE is the main risk factor for EA and it represents a precancerous lesion that might progress towards malignancy. The exact progression rate of BE to EA is still unknown, but, since EA is still one of the worst cancers to treat (Ferlay J et al 2014) it is mandatory to identify molecular biomarkers that might lead to early diagnosis. Since BE is the most important precursor for EA, screening protocols have been recommended, although there are currently no evidences that BE screening effectively reduces EA incidence and mortality (Fitzgerald RC et al 2014). In search of novel BE biomarkers, we provide evidence that hERG1 channels can be considered novel markers of progression in BE patients. In particular, we showed that hERG1 is over-expressed in BE, confirming data obtained from a previous study conducted by our group (Lastraioli E et al 2006). In the present thesis, we demonstrated that hERG1 potassium channel can be easily detected in BE samples through IHC analysis using a monoclonal anti-hERG1 antibody, which recognizes an extracellular epitope of the protein. We applied an immunohistochemical scoring method to evaluate hERG1 expression, based on the contemporary assessment of the percentage of labelled cells and the signal intensity, and we proved that hERG1 expression increases along BE progression to ED and EA. This was proven in separate cohorts of BE, ED and EA samples as well as in a subset of patients whose BE lesions progressed to ED or EA and for which matched BE and ED/EA samples were available. Performing a retrospective case-control study, we obtained the main translational result of this study, in which the association between hERG1 expression and development of adenocarcinoma was evaluated in samples from patients' biopsies collected at the diagnosis of BE. Patients enrolled in the study had a

follow up of at least 10 years, so that the progression to EA had been adequately monitored. Our data demonstrated a statistically significant association between hERG1 expression in BE patients and risk of progression to EA (odds ratio = 3.70, 95% CI: 1.40–9.82; P= 0.006). In other words, hERG1 expression identifies a group of patients whose lesions are suitable of progressing towards EA. Nevertheless, since the percentage of hERG1-positive progressed BE samples is not strikingly different from hERG1-positive not-progressed BE (although the statistically significant results reached), extreme caution should be applied, to take into account false-positive samples. We then tested whether hERG1 expression could be detected in different BE mouse models, in order to be exploited in the future for *in vivo* imaging and pharmacological studies. In particular, we confirmed hERG1 expression in the metaplastic cells arising in BE lesions of two different mouse models: a surgically-induced and a chemical-transgenic model. Furthermore, in one mouse in which BE progressed to ED, the scoring of hERG1 expression increased. Interestingly, we also showed that the percentage of mice developing BE after the surgical procedure greatly increased (from 36% to 100%) in transgenic mice over-expressing hERG1 gene in the GI tract. Although these results were obtained in a small set of animals, they might suggest a potential causative role of hERG1 in BE pathogenesis, a topic to be further studied in the future.

In the recent years it came to know that hERG1 plays a key role in several types of human cancer. In particular, hERG1 is a marker of advanced stage in colorectal cancers, contributing to identifying high risk TNM stage II patients (Lastraioli E et al 2004; Muratori L and Petroni G et al. 2016). In pancreatic and gastric cancers hERG1 is a prognostic biomarker also in early stages cancers, where it contribute to identify patients with worse prognosis (Crociani O et al 2014; Lastraioli E et al 2015). In the field of esophageal diseases, hERG1 channels might represent a progression factors that

identify high-risk BE patients and could therefore be useful for endoscopic surveillance in order to ensure a better follow up and early EA diagnosis. The aberrant expression of hERG1 in esophageal mucosa, even at early stages of esophageal cancerogenesis, could in turn modify cellular behavior switching on survival and proangiogenic signals, which in turn promote proliferation of BE metaplastic cells (Zhang Q et al 2014). To date, ED is the best histopathological progression marker but it is affected by several biases such as biopsy sampling error and subjective evaluation. For these reasons, other progression markers have been proposed over the years, such as aneuploidy and 9p and 17p loss of heterozygosity (LOH) (Weston AP et al 2001; Spechler SJ et al 2011; di Pietro M et al 2015). Recently, immunohistochemical evaluation of P53 has been proposed as a progression marker, although the wide variation in the expression as well as the high false-negative and false-positive rates limit its usefulness (reviewed in Arcangeli A et al 2015). In the present thesis, we provide evidence that immunohistochemical evaluation of hERG1 on biopsies obtained during endoscopic procedures might represent a valid and useful tool to better diagnose BE patients at high risk of progressing towards EA. Once validated, the possibility of including hERG1 channel to a panel of other BE progression predictive biomarkers could help to design the most useful surveillance or treatment protocol.

CHAPTER 2

The role of gonadotropins in the genesis of malignant diseases, in particular gynecologic cancers, is still controversial. In this chapter we focused on luteinizing hormone receptor (LHR) and its role in endometrial cancer (EC). The increased ability of EC cells to undergo local invasion and metastatic spread, promoted by the binding of LH to its receptor, was demonstrated by our group in an orthotopic/menopausal mouse model. To date, EC was studied *in vivo* producing xenograft and orthotopic mouse models. For this purpose we have generated a LHR transgenic mouse model that over-express hLHR in the female reproductive tract, in order to evaluating whether LHR over-expression is capable *per se* of inducing the development of EC. If these transgenic mice will be proven to be a good model for EC establishment, they will be used in the future to develop and test appropriate therapeutic interventions.

1. INTRODUCTION

1.1 GONADOTROPINS

Gonadotropic hormones, such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are glycoprotein hormones released in a pulsatile manner from the hypophysis and act together to regulate gonadal function. The releasing of gonadotropins is regulated by the hypothalamus through the pulsatile secretion of gonadotropin-releasing-hormone (GnRH). Biological actions of LH and FSH include

stimulation of the maturation and function of the testis and ovary and regulation of gametogenesis and steroidogenesis (Catt K et al 1991). Testicular LH receptors (LHR) are expressed during fetal life, postnatally, at puberty, and adult life (Dufau ML 1998). In testis, LH acts on the Leydig cells to promote the production and secretion of testosterone (Segaloff and Ascoli 1992). In the ovary, LH promotes the maturation of follicular cells. After the initial inductive effect of FSH on LHR expression in the small follicles, LH enhances the subsequent stages of follicular development and steroidogenesis in granulosa and luteal cells (Richards JS et al 1988; Menon et al 2005). The LH peak promotes the ovulation by promoting the rupture of the preovulatory follicle and the release of the ovum. During fetal development, LHR is not detectable in the fetal ovary but is expressed in early neonatal life (Dufau ML 1998). FSH stimulates follicular maturation and estrogen production by granulosa cells in the ovary (Shemesh M et al, 2001). All the glycoprotein hormones are heterodimers containing a common α -subunit and dissimilar β -subunits that confer biological specificity on the individual hormones. Gonadotropin receptors bind the intact heterodimeric hormone, but the individual hormone subunits are devoid of binding activity (Catt K et al 1991). Another gonadotropin is the human chorionic gonadotropin (hCG), which is synthesized and secreted by syncytiotrophoblastic cells of the placenta from the time of implantation, maintains the secretions of estrogen and progesterone by the corpus luteum during pregnancy. LH and (hCG) are structurally similar and share the same receptor (LHR). Due to its ability to bind both LH and hCG with high affinity, LHR is also known as LH/hCG receptor.

1.2 LH RECEPTOR (LHR)

The luteinizing hormone receptor (LHR) play a crucial role in the regulation of reproductive functions including ovarian steroidogenesis, ovulation in the female, and

testosterone production by the Leydig cells of testis (Ascoli et al 2002; Menon et al 2005; Dufau ML 1998).

LHR was demonstrated to be expressed in the follicles and corpus luteum in the ovary and the Leydig cells of testes (Ziecik AJ et al 1986). The presence of LH/hCG receptors in the uterus was next demonstrated in rabbit, mouse and human (Sawitzke AL et al 1991; Mukherjee D et al 1994; Reshef E et al 1990). To date it is clear, that LH/hCG receptors are widely distributed in non-gonadal tissues including the female and male reproductive tract (oviduct, uterus and male accessory sex organs), placenta, mammary gland, brain, adrenal cortex, T lymphocytes and urinary bladder (Ziecik AJ et al 2007).

1.2.1 Structure

LHR is a single polypeptide chain with a structure that makes it a member of the rhodopsin/ β_2 -adrenergic receptor subfamily of G protein-coupled receptors (GPCRs) and it is encoded by a single gene located in the short arm of chromosome 2 (2p21) (Ascoli et al 2002; Rousseau-Merck MF et al 1990). This gene is about 80 kb in size and each consists of 10 introns and 11 exons (Atger et al., 1995; Tsai-Morris et al., 1991). Exons 1-10 and a portion of exon 11-encode the N-terminal cysteine-rich region, all of the leucine-rich repeats (LRRs), and the N-terminal end of the hinge region of the extracellular domain. The remainder of exon 11 in LHR encodes the seven transmembrane helices, three extracellular loops, three intracellular loops, and an intracellular C-terminal tail (Puett D et al 2005). In agreement with the orientation of other GPCRs, we can recognize three distinct domains in the LHR, a large extracellular N-terminal domain, a central region containing seven transmembrane segments connected by three intracellular loops and three extracellular loops and a short intracellular C-terminal tail (Figure 1).

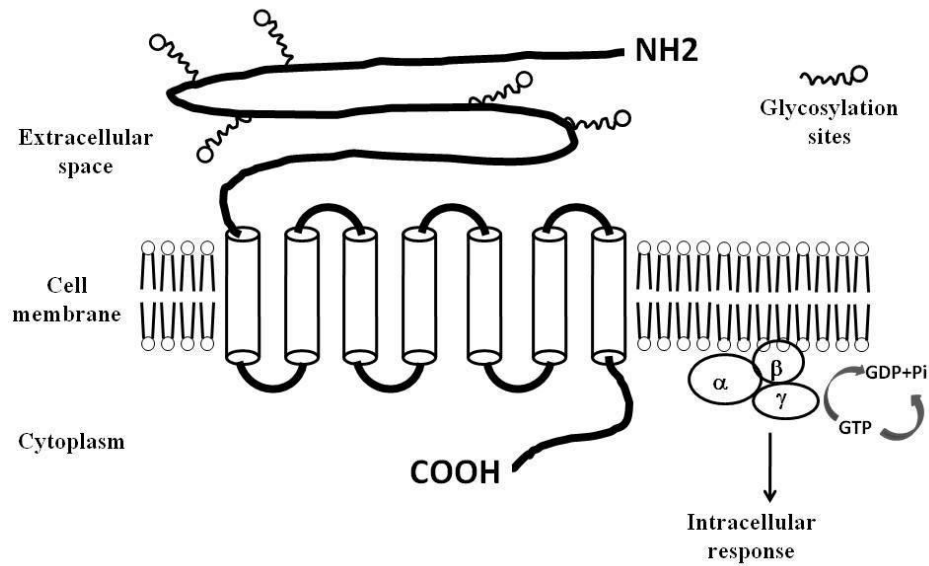


Figure 1: Stylized representation of the LH receptor with its intracellular, extracellular and transmembrane portions.

The long extracellular domain contains about 341 residues and binds LH and hCG with high affinity (Wang Z et al 1993). The exodomain contains leucine-rich repeats (LRRs) of 22–29 amino acids, that forms a α/β horseshoe fold, which is important for the protein-protein interactions (Dufau ML et al 1998; Enkhbayar P et al 2004). The LRRs domains of the LH, FSH, and TSH receptors share sequence similarities of about 43% within exons 2–8. However, the similarity diminishes at the N- and C-terminal domains of the extracellular region with the presence of amino acid inserts in the FSH and TSH receptors, suggesting that these regions may be important for denoting hormone specificity (Dufau ML et al 1998). Moreover, six potential glycosylation sites have been identified in human LHR which are required for the proper folding of the protein (Menon KMJ et al 2012).

The LHR receptor is palmitoylated on two cysteine residues in the C-terminal tail. Receptor palmitoylation is believed to provide two anchoring sites for the cytosolic tail onto the plasma membrane (Moench SJ et al 1994). Although abrogation of the palmitoylation sites does not reduce the ability of the receptor to bind ligand or mediate

activation of adenylate cyclase or inositol phosphate breakdown, the absence of palmitoylation increases the LH/hCG-induced internalization of the receptor. It is reasonable to speculate that the receptor, after binding hormone, might undergo depalmitoylation to facilitate its internalization (Bradbury FA et al 1999; Menon KMJ et al 2004). The C-terminal sequence also contains several serines and threonines that are amenable to phosphorylation by protein kinase A. In addition, the intracellular portion contains consensus sites for protein kinase C phosphorylation, and tyrosine kinase phosphorylation (Dufau ML et al 1998).

The seven transmembrane helices are connected by three extracellular and three intracellular loops. The receptor contains cysteine residues in the first and second extracellular loops, as do other members of the superfamily. These residues form an intramolecular disulfide bridge that stabilizes the helical seven-transmembrane structure (Dufau ML et al 1998).

1.2.2 LHR signaling

The signal transduction pathways of the gonadotropin receptors include activation of both the adenylate cyclase and phospholipase C systems. Like other members of GPCR family, the intracellular loops of LHR interact with G proteins. G proteins are heterodimeric complexes made up of α , β and γ subunits, able to bind GTP and GDP. Binding of LH to LHR results in a conformational change in the receptor that is transmitted to the G protein and lead to α subunit cycle from inactive (GDP-bound) to active (GTP-bound) (Digby GJ et al 2006). Active G protein heterotrimers dissociate into α -GTP and $\beta\gamma$ subunits that interact with other intracellular proteins to continue the signal transduction cascade. LH mediates its action on the endometrial cells via two intracellular second messenger systems. The first one involves the activation of adenylate cyclase and the subsequent production of cAMP and the second one the

stimulation of phospholipase C (PLC) and the activation of protein kinase C (PKC) (Ziecik AJ et al, 2007) (Figure 2).

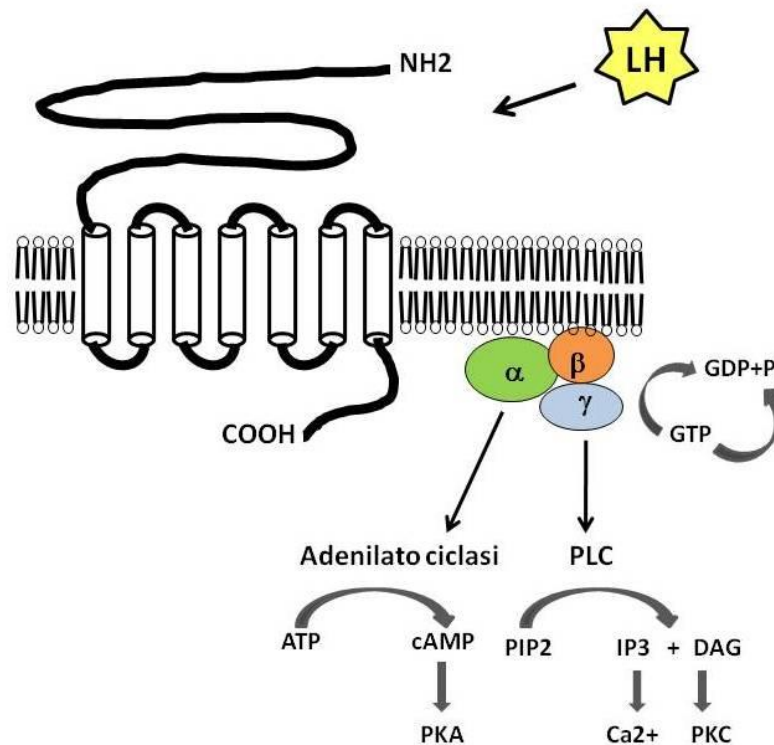


Figure 2: Representation of the main signaling pathways promoted by the binding of LH to LHR. The activation of LHR leads to the activation of PKA and phospholipase C pathways.

LHR expressed in target tissues is known to respond to a physiological concentration of LH or an equivalent concentration of hCG by mediating the activation of adenylate cyclase (Menon KMJ et al 1974). At superphysiological concentrations, LH/hCG has been shown to stimulate phospholipase C, leading to inositol phosphate breakdown and the consequent production of inositol trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ is a stimulator of calcium mobilization, whereas DAG is a potent stimulator of protein kinase C (PKC) (Nishizuka Y et al 1984).

Increased amounts of second messenger were associated with increased concentrations of endometrial cyclooxygenase (COX-2) and its metabolite PGF. The physiological significance of PGF production during the postovulatory phase of the oestrous cycle is

not known. Secretion of PGF at this early stage of the cycle may be necessary for the movement of the early embryo from the Fallopian tube to the uterus (Shemesh M et al, 2001). LH may also act through cAMP to regulate progesterone synthesis and its metabolism (Bonnamy PJ et al, 1989).

LH activates both the cAMP and phospholipase C pathways and the effect of LH on both pathways at each stage of the cycle is correlated with the amount of LHR present in the tissue. Activation of these signalling pathways is associated with an increase in the expression of cyclooxygenase and production of PGE in the myometrium (Shemesh M et al, 2001). In myometrium high concentration of LHR, induced by estradiol, resulting in an increase in cAMP may serve to allow the relaxing of the uterus during the luteal phase (Shemesh M et al, 2001).

The LH/hCG receptor has also been shown to mediate activation of the mitogen-activated protein kinase (MAPK) and Janus kinase-signaling pathways (Srisuparp S et al, 2003, Carvalho CR et al, 2003). It has been suggested that LH-promoted MAPK stimulation may result in the desensitization of LH-stimulated steroidogenesis in granulosa cells (Amsterdam A et al, 2002; Menon KMJ et al 2004).

1.3 MOUSE MODELS

Genetically engineered mouse (GEM) models have significantly contributed to the understanding of cancer biology and its molecular mechanisms. They have been proven to be useful in validating gene functions, identifying novel cancer genes and tumor biomarkers, gaining insight into the molecular and cellular mechanisms underlying tumor initiation and multistage processes of tumorigenesis, and providing better clinical models in which to test novel therapeutic strategies. However, mice still have significant limitations in modeling human cancer, including species-specific differences and inaccurate recapitulation of de novo human tumor development (Cheon DJ et al

2011). Mice share with humans many anatomical, cellular, and molecular characteristics that are known to have critical functions in cancer, such as an immune system, maternal effects in uterus, imprinting of genes, and alternative splicing.

1.4 GENETICALLY ENGINEERED MOUSE MODELS (GEM)

With the availability of the complete sequence of the mouse genome, technology to manipulate the mouse genome, and well-defined inbred strains, the ability to engineer mice is impressive. Experiments can be undertaken to assess the outcome when the function of a gene is lost, mutated, underexpressed, or overexpressed in the appropriate cell types *in vivo* (Walrath JC et al 2010). Mutagenesis studies in the mouse have identified new cancer-causing mutations that can then be confirmed in studies of human cancers.

1.4.1 Loss of gene function in GEM

Studying the loss of function of genes provides insight into understanding the biological functions for which the protein product is required. Loss-of-function studies most commonly use “knockout” strategies to remove the gene of interest by engineering constitutive or conditional deletions in the gene. For genes that span large genomic regions, deletion of the first few exons encoding the start codon is often sufficient to block transcription or translation into a functional protein product. The use of knockout strategies have been critical in understanding cause and effect relationships in cancer development, and can be applied to the assessment of many gene classes, including oncogenes, tumor suppressor genes, and metabolic (“housekeeping”) genes (LePage DF and Conlon RA 2006).

Knockout mouse models: Conventional knockout vectors contain a positive selectable marker, usually neomycin (Mansour et al 1988). This allows the replacement of specific

genes with Neo through homologous recombination between the targeting vector and the cognate sequence in the recipient clonal embryonic stem (ES) cell genome after the vector is transferred into these cells by electroporation. Only neomycin resistant ES cells generated by homologous recombination can grow under selection. The first step of this technique involves the isolation of a ES cell line that contains the desired mutation. The second step is to use these ES cells to generate chimeric mice that are able to transmit the mutant gene to their progeny. This is accomplished by injecting ES cells that contain the desired targeted mutation into a blastocyst. These blastocyst are then surgically transferred to a pseudopregnant foster mother to allow the embryos to come to term. To facilitate isolation of the desired progeny, the ES cells and recipient blastocysts are derived from mice with distinguishable coat-colour alleles (for example, ES cells from agouti brown mice and blastocysts from black mice). The extent of the contribution of ES cells to the formation of the chimeric mouse can be evaluated by visual assessment of coat-colour chimerism (Figure 3). ES cell contribution to the germline can be evaluated by observing the coat colour of the progeny that is derived by breeding the chimeric mouse with black wild type mice (Capecchi MR 2005).

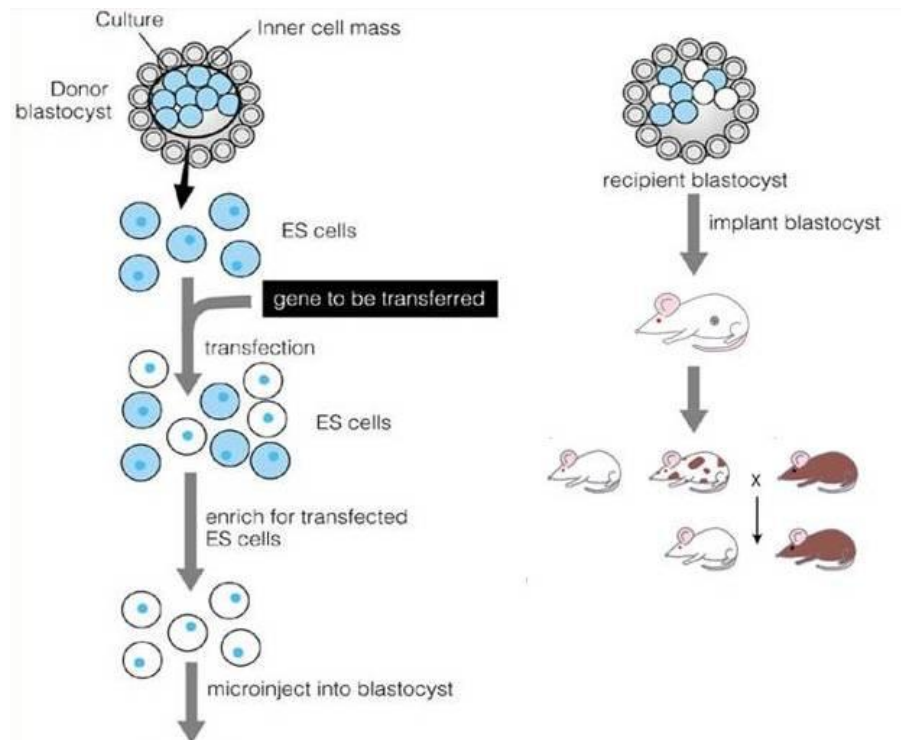


Figure 3: Generation of mouse germline chimaeras from embryonic stem cells that contain the desired targeted mutation.

With conventional knockouts, loss of a vital gene can often lead to embryonic lethality or adult sterility, making it impossible to study the gene in the disease context. In addition, ablation of the gene of interest in the entire body does not mimic spontaneous tumorigenesis in humans, where tumors evolve in a wild-type environment, and the timing of gene loss may be a critical factor in disease development (Walrath JC et al 2010).

Mouse conditional gene mutations: to circumvent conventional knockout limitations, sophisticated conditional genetic engineering technology has been developed to create systems where genetic events can be tightly controlled spatially and temporally such as Cre/loxP system and TET system.

Cre/LoxP system: The Cre/loxP system mediates site-specific DNA recombination and was originally described in bacteriophage P1. Bacterial Cre enzyme is site-specific recombinase that catalyze specific recombination between defined 34 bp DNA-

sequences (*loxP*). In the presence of Cre protein expression any DNA sequence flanked by two *loxP* sites will either be excised (if *loxP* sites are in same orientation) or inverted (if *loxP* sites are in opposite orientation) (Figure 4) (Stricklett PK et al, 1999). A gene flanked by 2 *loxP* sites is called “floxed gene”.

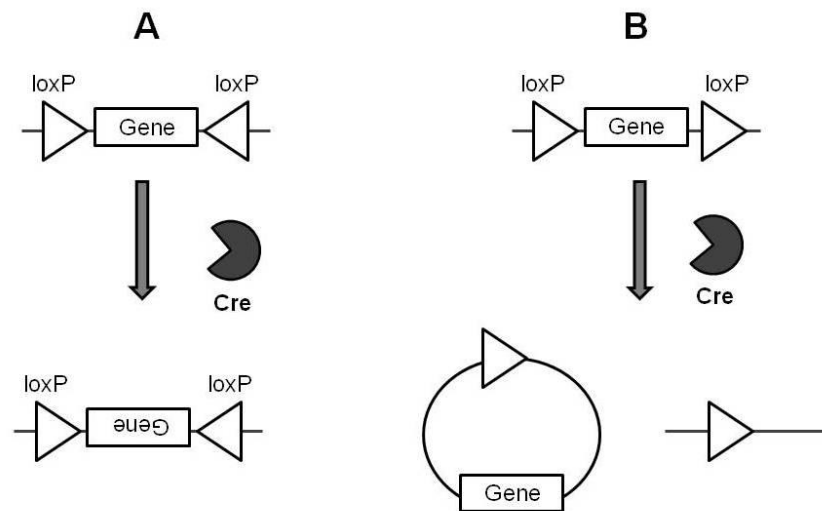


Figure 4: Schematic of Cre-mediated recombination of a gene fragment flanked by *loxP* sites. The gene is either excise or inverted if the *loxP* sites are in the same or opposite orientation, respectively.

In most of the studies a floxed STOP sequence is inserted 5' to a reporter gene. Upon Cre-mediated recombination, the STOP sequence is excised allowing the expression of the reporter. A major advantage of the Cre/*loxP* system lies in its relative simplicity. First, no cofactors are required for Cre activity. Second, *loxP* target sites are small and easily synthesized. Third, Cre is a very stable protein. Finally, and most important, it is easy to generate DNA constructs with any promoter of interest driving Cre expression. This permits controlling the tissue site, and possibly the timing, of Cre expression and resultant gene disruption. By temporally and spatially controlling expression of the recombinase, it is then possible to temporally and spatially control deletion of the gene of interest, overcoming interference from developmental abnormalities and lethality (Branda CS and Dymecki SM; 2004). Mice carrying the Cre recombinase under control

of a tissue-specific promoter are crossed with mice carrying the gene of interest flanked by loxP sites to conditionally knockout the gene in a specific tissue or at specific times during development.

TET system: The tetracycline-controlled transcription activation system was shown to function as an efficient genetic switch in a variety of eukaryotic cells, including mammalian cells. It also allows effective control of gene activities at the level of the organism (Baron U et al 1997). This system is composed of a transactivator and an effector. One of the key components of the tet system is the tetracycline-controlled transactivator (tTA), a fusion protein between the DNA-binding domain of the repressor of *Escherichia coli* tetracycline resistance operon (Tet Repressor Protein) and a C-terminal portion of the herpes simplex virion protein 16 (VP16) that contains domains capable of activating transcription (Baron U et al 1997). The tTA binds to the tetracycline operator (tetO) that controls the activity of the human cytomegalovirus promoter (CMV promoter) driving conditional gene expression. That system is named Tet-Off system because in the presence of tetracyclin, tTA is prevented from binding to tetO and thus transcription is abolished. In the absence of the effector tetracycline, tTA will activate transcription from a suitably engineered promoter by binding to an array of tet operator sequences positioned upstream. In the Tet-On system, the tetracycline-repressor has been mutated (rtTA) such that it is only in the proper conformation for association with tetO when it is bound to tetracycline, thus inducing expression of the gene in the presence of drug (Stricklett PK et al, 1999).

1.4.2 Gain of gene function in GEM

Mouse constitutive transgenic models: gain-of-function studies are often used to study oncogenes in mouse models. Transgenic or knockin animals constitutively overexpressing an oncogene can be used to study how the oncogene drives

tumorigenesis *in vivo*. Transgenic animals have been very useful in studying many oncogenes. Transgenic animals are created by the pronuclear injection of transgenes directly into the pronucleus of fertilized oocytes, followed by implantation into pseudopregnant females (Macleod KF, Jacks T et al 1999; Porret A et al 2006) Briefly, the main steps of this technique include: the coupling of females, treated with FSH and hCG, with fertile males; their sacrifice for the removal of zygotes, the injection of the construct, containing the gene/cDNA of interest, into the male pronucleus and the subsequent reimplantation of survived embryos in foster females (females coupled with sterile males ready to carry a pregnancy). The transgene is randomly incorporated into the genome and thus can incorporate into a gene necessary for development or fertility, causing deleterious effects and limiting the usefulness of the transgenic model. Furthermore, the epigenetic regulation of gene expression in the region surrounding the transgene integration can affect transgene expression levels and often result in silencing. Therefore, multiple founders must be screened to confirm adequate and specific expression of the transgene (Figure 5).

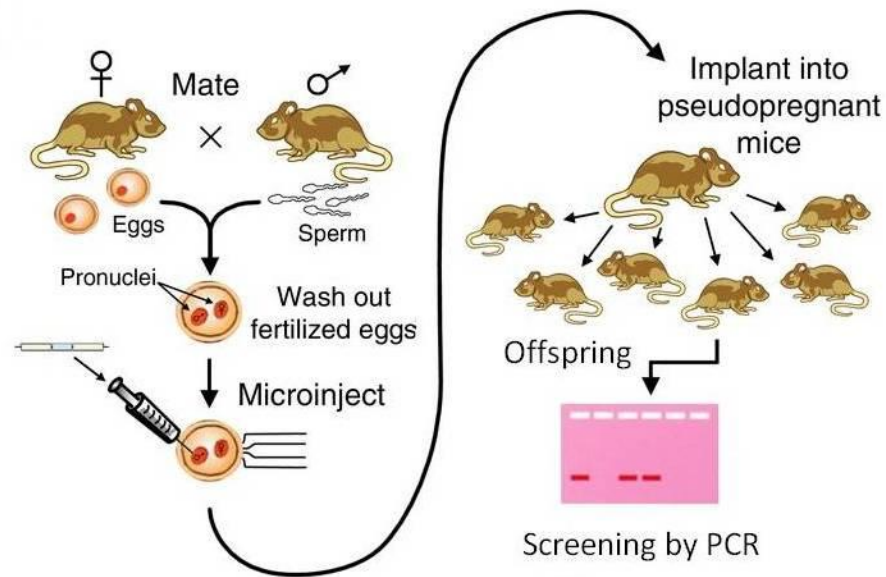


Figure 5: Generation of transgenic mice. In order to express a transgene *in vivo*, the investigator first makes a construct containing the transgene being evaluated. Then fertilized eggs are washed out of the oviducts of female mice. They are then microinjected under direct visualization and implanted into the uterus of pseudopregnant female mice. The genotype of the pups that are produced is evaluated in tail biopsy–derived DNA using PCR reactions (modified picture from Elias JA et al 2003).

To circumvent transgenic limitations associated with random insertion, knockin mice are created by inserting a gene of interest into a specific region of the genome using homologous recombination techniques, much like those used when creating knockouts. The *Rosa26* locus is commonly used as an insertion site for knockin animals because it is devoid of essential genes and allows for good expression of the transgene (Friedrich G and Soriano G 1991). While transgenics have the potential for multiple insertions of the transgene, knockin animals carry only one copy of the transgene. This approaches can also be used to replace a normal gene copy with a mutated version of the gene to examine specific mutational events in the context of normal control of the gene (Lang GA et al 2004).

Mouse conditional overexpression models: transgenic and knockin expression of deleterious genes may lead to lethality, sterility, and developmental defects that impede study of the gene of interest in cancer, as seen with many conventional knockouts. Therefore, spatial and temporal control of transgene and knockin expression may be necessary to circumvent these limitations. Conditional transgenics and conditional knockins can be created using tissue-specific promoters to constitutively drive expression or created by inserting a strong translational and transcriptional termination (STOP) sequence flanked by *loxP* sites in between the promoter sequence and the gene of interest (Lakso M *et al.*, 1992). Examples of commonly used STOP cassettes are the lox-STOP-lox in which multiple STOP sequences are arrayed between *loxP* sites (Jackson EL *et al.*, 2001) and the NEO-STOP cassette in which the neomycin resistance gene and a STOP sequence is inserted between *loxP* sites (Dragatsis I and Zeitlin S, 2001). The presence of the STOP sequence blocks transcription of the gene of interest. However, in the presence of Cre recombinase, the STOP cassette is removed, allowing expression. Since gene expression is dependent on excision of the STOP cassette and recombinase expression, gene expression can be spatially, temporally, and inducibly controlled with the Cre systems (Stricklett PK *et al.*, 1999).

1.5 MOUSE MODELS FOR GONADOTROPINS AND THEIR RECEPTOR

The main target of gonadotropins in females is the ovary, maturation of which is triggered by LH and FSH at puberty. It is largely agreed and confirmed by animal models lacking FSH, LH or their receptors that the gonadotropins are not critical for the prenatal female uro-genital development. Nevertheless, loss-of-function mutations in any of the gonadotropins or their receptors lead to delayed or interrupted progression of puberty, hypogonadism and consequently infertility. The potential role of gonadotropins in initiation and/or progression of ovarian tumors has been discussed for

decades. Intriguingly, in genetically modified animals both excess and lack of gonadotropin action have been linked with tumorigenesis including germ cell, sex-cord and surface epithelial tumors (Peltoketo H et al 2011). To study the roles of only FSH in mammalian reproductive physiology, Kumar and coll. developed FSH β null mice and hence lack a FSH heterodimer (Kumar TR et al 1997). Subsequently, using transgenic mouse model overexpressing hCG it was demonstrated that female mice are very sensitive to tumorigenic effects of the elevated LH/hCG action, and consequent elevation of ovarian steroidogenesis. A multitude of tumours and altered endocrine functions were observed in gonads, pituitary gland, mammary gland and adrenal cortex of these animals (Huhtaniemi I et al 2005). Indeed, LH knockout males and females were infertile and demonstrated reduced size testes and accessory glands, consistent with decreased serum and intra-testicular testosterone levels, and pituitary serum FSH levels were unaffected (Ma X et al 2004). In TG mice expressing the mouse inhibin α -subunit promoter/SV40 T-antigen fusion gene elevated LH levels likewise act as tumour promoter and induce gonadal and adrenal tumorigenesis (Mikola M et al 2003). In addition, mice deficient of the inhibin α -subunit gene develop mixed granulosa/Sertoli cell tumours in the presence of LH and FSH (Kumar TR et al 1996). The LHR knockout mouse model, developed by Huhtaniemi group, allowed to identify the specific LH-dependent steps of male and female sexual differentiation and adult gonadal functions. In particular, it was demonstrated that in each sex, the intrauterine sex differentiation is independent of LH action, but it has a crucial role postnatally for attaining sexual maturity (Zhang FP et al 2001). Overall, the loss and gain-of-function mutations affecting LH/hCG action in genetically modified mice have greatly expanded our knowledge about functions of the regulation of gonadal function. However, the rather robust TG and KO models currently available do not fully simulate

the genetic aberrations detected in human gonadotrophin action and in gynecological diseases.

1.6 EDOMETRIAL CANCER MOUSE MODELS

The endometrium is a classical hormone-dependent tissue and most of the endometrial adenocarcinomas are hormone-dependent tumors. A high percentage of these tumors express the estrogen, progesterone and LH receptors. Investigators have, therefore, aimed at targeting steroid hormone receptors by the development of novel sub-stances. For testing and characterization of these new substances appropriate models have to be available. Moreover, mouse models historically have proved to be particularly useful in studying the effects of developmental exposure to hormones, particularly estrogens. The neonatal mouse has been proposed as a model for hormonal carcinogenesis of the endometrium. Particular, CD-1 mice appear to be an excellent model to test for developmental toxicity of estrogens, thereby evaluating the capacity of test substances to induce hormonedependent endometrial cancers. CD-1 mice respond to treatment with N-methyl-N-nitrosourea (NMU) or estradiol with the development of endometrial cancers within 30 weeks. This process is significantly accelerated if mice are treated with both agents simultaneously (Niwa K et al 1991; Vollmer G et al 2003). In comparison to human endometrial carcinogenesis, histopathological examinations revealed that these tumors develop from various preneoplastic, hyperplastic lesions, resembling the human situation (Niwa K et al 1991). For the development of atypical hyperplasia the cooperative action of estradiol and NMU is favorable (Niwa K et al 1996).

As previously described, a significant rate of PTEN gene mutations has been reported for human endometrial adenocarcinoma (Risinger JI et al 1997). Unlike the human situation, in pten + / - -mice neoplasia of the skin and brain were notably absent,

whereas the observed changes in the endometrium were very consistent (Podsypanina K et al. 1999). In order to better understand the biological role of this tumor suppressor gene, transgenic PTEN knock-out mice have been created. A $Pten^{-/-}$ mutation is lethal presumably due to defective chorio-allantoic development but surprisingly, the mutation of one allele is sufficient to cause neoplasia in multiple organ systems in these $pten + / -$ mice (Suzuki A et al 1998). Stambolic et al. proposed $pten + / -$ mice as a model for endometrial adenocarcinomas which develop in women with unopposed estrogen stimulation. These patients rather commonly suffer from loss of heterozygosity at the PTEN locus and/or mutations in all stages of endometrial hyperplasia (Stambolic V et al 2000). $Pten +/-$ mice represent a promising endometrial cancer model, because of the similarity to hereditary human endometrial cancer (Vollmer G et al 2003).

Moreover, xenograft mouse models have been used in preclinical studies for their comparatively low cost and rapid, predictable tumor growth. An orthotopic xenograft mouse model of EC was developed in our lab in order to better study the capability of EC cells to undergo local invasion and metastatic spread (Pillozzi et al 2013). Pillozzi et al. evaluated the effect of LHR on invasiveness of EC, using Hec1A cells stably transfected with, and hence over-expressing, the human LHR (Hec1A-LHR cells). The model consists in athymic nu/nu mice which received an implant of very small tumor block in the uterine wall. The tumor blocks we implanted were obtained from subcutaneous masses obtained after injection of either Hec1A-LHR or Hec1A cells (not expressing LHR). Athymic nude mice are a very good model for this aim, since they display severe deficiencies in reproductive function such as delayed ovulation and gonadotropins are totally absent. In order to make the model closer to the condition of menopause, in which there are high levels of circulating LH, the mice were treated daily with high doses of LH. The use of such model allowed to conclude that the over-expression of the LHR on the membrane of EC cells, is critical to make EC cells to

become more aggressive, and capable of invading the myometrium as well as surrounding organs, and the concomitant over-expression of the LHR and high levels of serum LH, make EC cells capable of reaching the lung and give rise to lung metastases (Pillozzi S et al 2013).

2. MATERIALS AND METHODS

2.1 Generation of LHR transgenic mice

In order to assemble the construct, the luciferase gene was amplified from pGL4.51[Luc2/CMV/Neo] vector (Promega) and the hLHR gene from Hec1A cells by polymerase chain reaction (PCR). For both the PCR we used Phusion High-Fidelity DNA polymerase (Finnzymes, New England Biolabs). The reactions were made using the touchdown protocol starting from 70°C with a 2°C decrease each 4 cycles, till 52°C. The mogp-1 promoter was amplified by PCR from BL-1A stem cells derived from SV129 mice. The reaction was made using Expand Long Template PCR system (Roche) and the PCR program starts with an incubation at 94°C for 2 min, followed by 28 cycles, each involving denaturation at 94°C for 10 s, annealing at 60°C for 30 s, and extension step at 68°C for 2 min.

The 8000-bp mogpLuc2AhLHR construct was excised from the pBluescript SK+ vector and it was microinjected into the male pronucleus of fertilized zygotes of FVB mice. Fertilized zygotes were obtained from mating of FVB mice and implanted into pseudopregnant FVB females. The procedure was done at LIGeMA (University of Florence, Italy), following standard protocol. Resulting puppies were screened by PCR analysis of DNA extracted from tails. Two mice founders were found, one male and one female. Transgenic mice were maintained in heterozygous in FVB background. Mice were kept in plastic cages and maintained under conventional conditions at LIGeMA, University of Florence.

2.2 Screening PCR of transgenic mice

Transgenic mice were genotyped by end point PCR analysis of genomic tail DNA. The DNA was extracted using the Chelex extraction protocol by incubation of a 0.2 cm of mice tails in 150 μ l of 10% (w/v in water) Chelex 100 resin (BIO-RAD) for 4 hours at 56°C and 30 minutes at 98°C. The presence of the hLHR gene was checked on mouse tail genomic DNA by amplification of a 350 bp fragment using the forward 5' GGCTGAAGAGCCTGATCAAATACA 3' (ScrLucLHRup) and reverse 5' CGCATGTTAGCAGACTTCCTCT 3' (ScrLucLHRdn) primers at final concentration of 300 nM. These primers recognize a region into a luciferase sequence. The reaction was performed using the PCR SuperMix (Platinum PCR SuperMix, Invitrogen). PCR program start with denaturation at 94° for 2 min, followed by 35 cycles at 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec, and a final extension cycle at 72°C for 10 min.

2.3 RNA extraction and reverse transcription

Uterus and ovary of both wild type and transgenic animals has been dipped in TRIzol Reagent (Invitroge) and homogenized to isolate total RNA according to manufacturer's protocol. RNA concentrations were determined using spectrophotometer by measuring the absorbance at 260 nm (A260). The ratio of the readings at 260 nm and 280 nm (A260/A280) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein and phenol. Pure RNA has an A260/A280 ratio of 1.8–2.1. Before reverse transcription, RNA integrity was evaluated by agarose gel (1%) electrophoresis.

Reverse transcription was done from 1 μ g of RNA using 200U/ μ L of SuperScript II Reverse Transcriptase (Invitrogen), adding for each sample 500 μ mol/L of deoxyribonucleotide triphosphate (dNTP) (Invitrogen) and 15 ng/ μ L of random primers (Invitrogen). The reaction starts preparing a mix with:

- 1 µg of RNA
- 2 µl of Random Primers (diluted 1:20 from the stock solution)
- 1 µl of dNTPs (10mM)
- ddH₂O till a final volume of 12 µl

The mix was incubated in a thermal cycler at 65°C for five minutes, and then immediately placed on ice for five minute. Then, the following was added to the tube:

- 4 µl of Buffer 5X First Strand
- 3 µl of ddH₂O

The mix was heated at 25 °C for 2 min and than added with the SuperScript II. The mix was incubated as follows:

<u>Temperature (°C)</u>	<u>Time (min)</u>
25°C	10
42°C	50
70°C	15

2.4 Real time PCR

hLHR expression was evaluated by real-time quantitative PCR (qPCR) using 1 µL of cDNA. QPCR involves the use of fluorescence to detect the threshold cycle (Ct) During PCR. When the level of fluorescence signal gives over the background and is in the linear portion of the amplified curve. This Ct value is responsible for the accurate quantisation of qPCR. We used Sybr Green Master Mix Kit (Applied Biosystem). The primer sequences for hLHR are: 5'-TGCCTACCTCCCTGTCAAAG-3' forward primer; 5'-TTGAGGAGGTTGTCAAAGG-3' reverse primer. β-actin was used as housekeeping gene. The primer sequences for β-actin are: 5'-GGGGTGTGTAAGGTCTAAA-3' forward primer; 5'-

GATCTGGCACCCACACCTTCT-3' reverse primer (Fiore A et al 2013). All the primers were used at final concentration of 300 nM. PCR program start with an incubation at 95 °C for 10 min, followed by 40 cycles of amplification: denaturation at 95 °C for 15 s and annealing–extension step at 60 °C for 1 min. Each reaction was performed in triplicate. Melting curve analysis of the amplicons were performed to exclude the amplification of a specific products or primer-dimer artefacts. LHR expression values of the samples were normalized on Hec1A cells. The relative quantification of LHR gene expression was performed by the Δ Ct method.

2.5 Analysis of uterine morphometry

The reproductive tract of 12 female mice (3 wild type and 9 transgenic) was rapidly excised and fixed in 4% formaldehyde for 4 hours, transferred in a graded series of alcohol, embedded in paraffin, cut lengthwise at a thickness of 6 μ m and put on positive-charged slides to perform analysis of uterine morphometry and immunohistochemistry (IHC).

Samples were stained with Hematoxylin/Eosin standardized protocol. The uterine radius was measured from the serosal side to the apical surface of the luminal epithelium. The muscle layer was considered the inner circular layer. The luminal epithelial height was measured from the basement membrane to the apical surface. All measurements were performed using a light microscope (Leica DMR, Germany) equipped with Leica DC Viewer and Leica Qwin. The measurements were taken from the slide that showed the uterine cavity and two measurements per area were analyzed.

2.6 Immunohistochemistry

After dewaxing and dehydrating the sections, endogenous peroxidase were blocked with 1% H₂O₂ solution in phosphate-buffered saline. Then, antigen retrieval was performed

by heating the samples in a microwave oven at 600 watt in citrate buffer pH 6.0 for 12 minutes. Samples were permeabilized with a 0,1% TRITON X100 in UltraVBlock solution (LabVision) and incubated overnight at 4°C with the primary antibodies: anti-c-myc (monoclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Ki67 (monoclonal antibody, Dako) and anti- α -SMA (monoclonal antibody, Dako), at a 1:100 final dilution. Immunostaining was carried out with a commercially available kit (PicTure-Max polymer Detection kit, Invitrogen) according to manufacturers's instruction. C-myc and α -SMA expression were evaluated with an estimate of the percentage of immunoreactive cells and samples were classified as positive when the percentage of labeled cells was greater than or equal to one. Ki67 expression was evaluated in three different areas of the section: percentage of stained cells in luminal epithelium, in glandular epithelium and in stroma.

3. AIMS OF THE STUDY

This study was aimed to investigate the role of LH receptor in the process of *in vivo* EC carcinogenesis. For this purpose we have generated a transgenic mouse model over-expression LHR in gynecological tract with the aim of determining whether LHR over-expression is capable of inducing the development of EC. Tissues of transgenic mice will be taken and evaluate.

This model will be further used evaluating the effect of LHR over-expression on the susceptibility to carcinogen agents. If the LHR transgenic model will be proven to be a good model for EC establishment it will be used for pharmacological tests.

4. RESULTS

4.1 Production of LHR constitutive transgenic murine model

In order to produce a LHR transgenic (TG) mouse model in our laboratory we previously produced a construct incorporating the hLHR cDNA under the control of Mogp-1 mouse promoter. Mogp-1 gene is active in the oviduct, ovary, uterus and vagina of mice (Miyoshi et al., 2002). At the 3' of the hLHR cDNA it was placed the sequence coding for the myc-tag. For the *in vitro* and *in vivo* assessment, we included the luciferase reporter gene in the construct whose synthesis occurs equimolar to LHR, due to use of the 2A peptide sequence, which is separated in two fragments during the translation (Tirichas et al., 2008). The 2A peptide is inserted in frame between the cDNA of LHR and the cDNA of Luciferase. All the characteristics of the final construct are represented in figure 6.

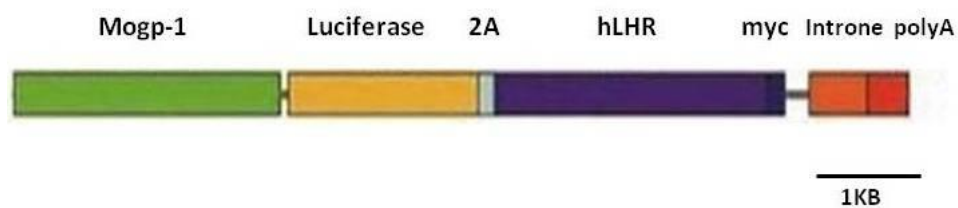


Figure 6: Picture of the construct used for the generation of a mouse model overexpressing LHR. The LHR and the Luciferase sequences are inserted in frame as a single cDNA, separated by a specific virus sequence (2A sequence), allowing the production of the two single proteins in an equimolar manner. 1 and 2 are the primers used for screening by PCR of founders mice.

The proper functioning of this construct was tested in *in vivo* experiments transfecting HEC1A endometrial cancer cells. The transfection was performed in duplicate using either the pBlueScript SK+ vector containing the complete construct

(mogpLuc2AhLHR) and the empty pBlueScript SK+ plasmid, used as negative control. Data obtained from the analysis with the luminometer showed an increase of RLU (Relative Light Unite) in cells transfected with mogpLuc2AhLHR construct, compared to those transfected with the empty vector (Figure7A), index of a proper functioning of the mogp-1 promoter and an appropriate production of luciferase. Moreover, in order to verify the proper hLH receptor placement on the plasma membrane we performed immunofluorecence experiments in HEC1A cells transfected with the vector containing mogpLuc2AhLHR construct. For this assessment we used anti-myc primary antibody as detailed in Materials end Methods. Although the low transfection efficiency the hLHR is properly placed on the membrane (Figure 7B, white arrows).

A

Vector transfected	RLU/40.000 cells
pBluescript SK+	13
pBluescript SK+	12
mogpLuc2AhLHR	3559
mogpLuc2AhLHR	2581

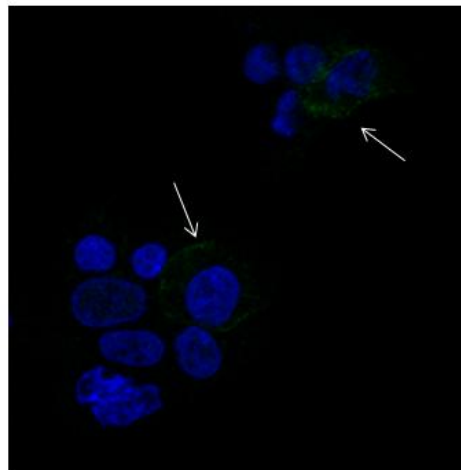
B

Figure 7: **A)** Data coming from the analysis to the luminometer after transfection of Hec1A cells. **B:** Confocal microscope images showing in green the presence of LHR in the membranes (Indicates with white arrows). Immunofluorescent staining with Ab I anti-myc, 1:100 and Ab II Alexa 488, 1:500. The nucleus are colored with DAPI.

Subsequently, the construct containing the cDNA of LHR was microinjected into the male pronucleus of mouse zygotes, which has been then reimplanted in the oviduct of fosters females. Briefly, the main steps of this technique include: the coupling of CD1 females, treated with FSH and LH, with fertile males; their sacrifice for the removal of zygotes, the injection of the construct into the male pronucleus and the subsequent reimplantation of survived embryos in fosters females (females coupled with sterile males ready to carry a pregnancy). Resulting puppies were screened by PCR analysis of DNA extracted from tails. Two out of five mice born from the reimplantation were found positive at the screening, one male and one female (respectively named as LHR-100 and LHR-200), and they were used as founders mice (Figure 8).

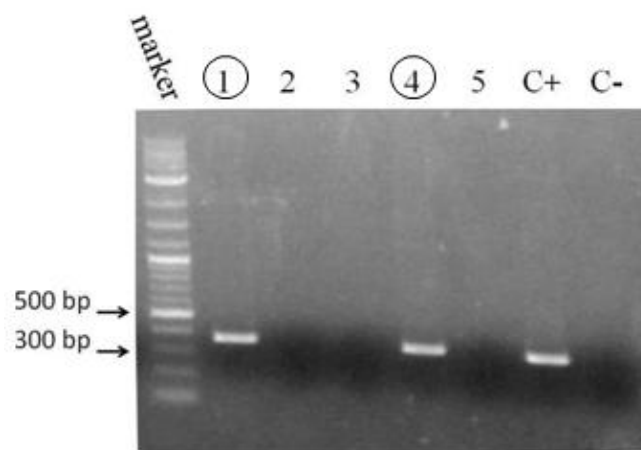


Figure 8: Electrophoretic analysis for the screening of transgenic mice shows the presence of 2 founders in lanes 1 and 4 (agarose gel). Samples in lanes 1-5. In lane 6, positive control. In lane 7, negative control.

4.2 Characterization of LHR transgenic mice

The two founders mice were mated with FVB wild type animals in order to maintain and breed hemizygous transgenic mice and to give origin at the two transgenic colonies: TG-LHR-100 and TG-LHR-200. In terms of number of puppies, no differences were found between transgenic mice and FVB wt animals (Figure 9), though

the males of the LHR-100 seems to have some fertility issues, because only one male of that line produced a litter.

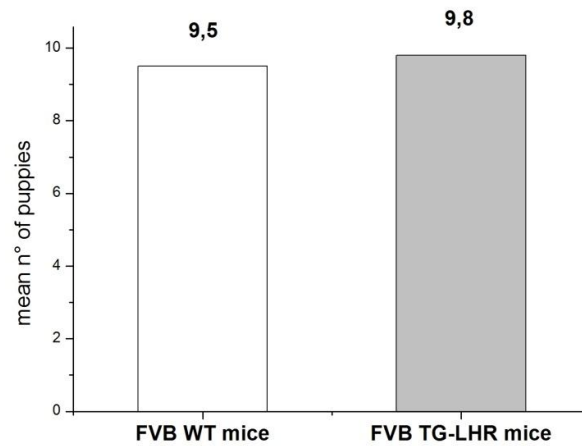


Figure 9: Column plot representing the minimal differences between the mean number of puppies of FVB WT mice and TG-LHR mice.

To evaluate the expression of hLHR gene in TG-LHR mice we sacrificed three transgenic mice and three FVB wt mice with the same age (< 12 months) and uterus, ovary, liver and spleen were taken. These organs were processed for RNA extraction and retrotranscription in cDNA (as detailed in Materials and Methods). RQ-PCR analysis showed that TG mice had hLHR mRNA high expression in the uterus, higher compared to wt controls. In particular, performing t-test analysis it was emerged that the different expression of hLHR between transgenic and wt animals was statistically significant ($p=0,018$)(Figure 10).

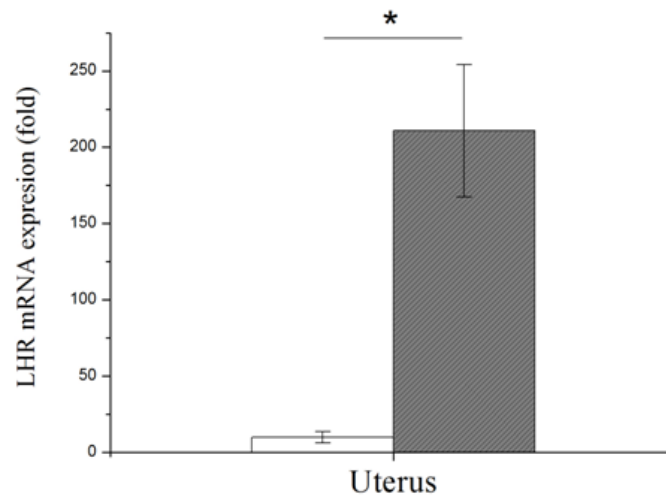


Figure 10: Graph representing LHR mRNA expression values in the uterus of TG-LHR-200 <12-months-old mice VS wt mice of the same age.

The expression of the transgene was found higher also in the liver of TG-LHR mice compared to the liver of wt animals (Figure 11). The different expression between transgenic and wt animals was statistically significant ($p=0,023$). This data may suggest the ectopic expression of the transgene.

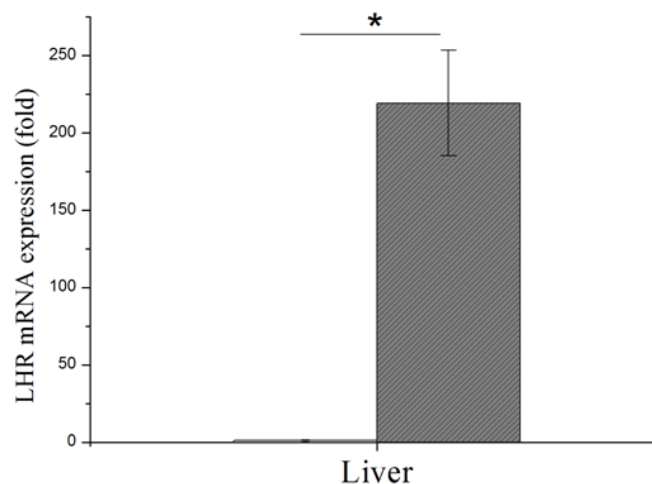


Figure 11: Graph representing LHR mRNA expression values in the liver of TG-LHR-200 <12-months-old mice VS wt mice of the same age.

Moreover, the expression of the hLHR mRNA was higher in the ovary and spleen of the TG-LHR mice compared to the wt animals, though the different expression between

transgenic and wt animals was not statistically significant (Figure 12). Although, normalizing the LHR expression value of the TG mice to the wt mice it emerged that the expression is higher in both ovary and spleen of transgenic animals.

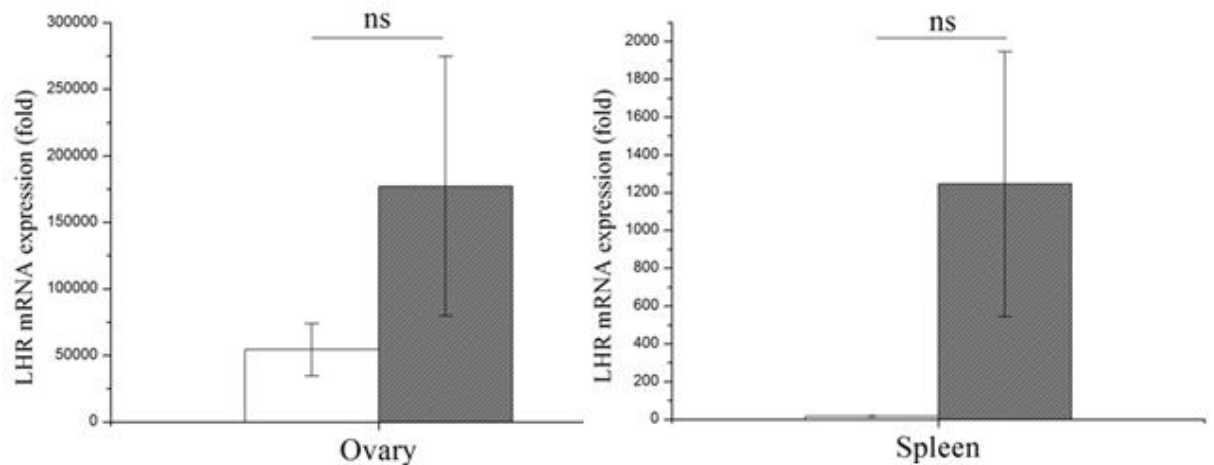


Figure 12: Graphs representing LHR mRNA expression values in the ovary (left panel) and spleen (right panel) of TG-LHR-200 <12-months-old mice VS wt mice of the same age. P values: ovary $p=0,33$; spleen $p=0,22$.

To confirm the over-expression of hLHR in TG-LHR mice we performed also an immunohistochemistry analysis on uterus slides of a 12-years-old TG mouse using anti-myc primary antibody (as detailed in Material and Methods). As shown in figure 10 the labeling is present in TG-LHR-200 mouse (Figure 10A) and in TG-LHR-100 mouse (Figure 10B), but it is absent in wild type mouse (Figure 10C), that confirm the presence of the LHR transgene in endometrium of TG mice.

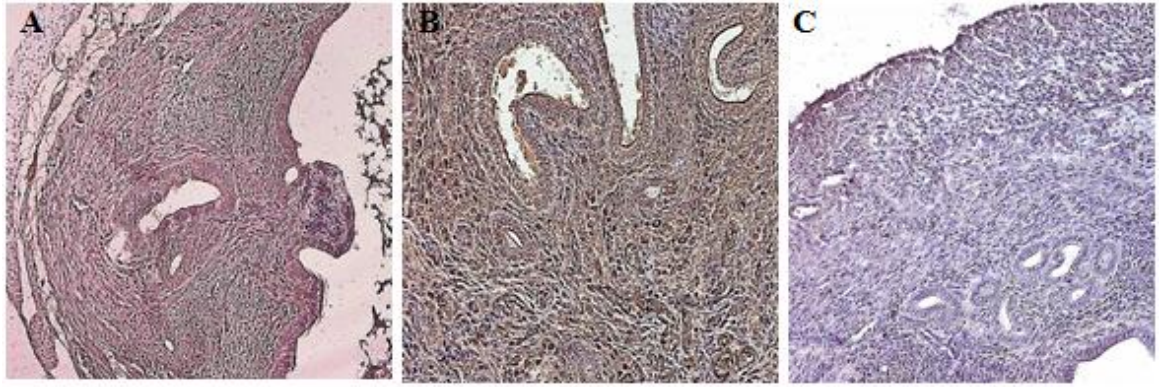


Figure 10: **A:** Graph representing LHR mRNA expression values in uterus and ovary of TG-LHR 3-months-old mice. The values were normalized on LHR expression of wt mice. **A,B,D:** Immunohistochemical analysis with anti c-myc antibody on representative samples of mouse uterus: c-myc is expressed in TG LHR- 200 (**A**), TG-LHR-100 (**B**), in **C** c-myc is absent (wt mouse). Magnification 20X.

4.3 Morphologic and morphometric characterization

For the histological characterization we sacrificed three 9-months old TG-LHR mice and the uterus were taken, formalin-included and paraffin-embedded (as in Material and Methods). 6 μ m-slides were stained with hematoxylin and eosin for the histological analysis. The observation at the microscopy (1,6X magnification) shown an increase in the dimension of the uterus of the three TG mice compared to wild type animals.

Moreover, it is worth noting that in 2 out of 3 of 9-months old transgenic animals is emerged an increase of the uterine cavity (LHR 108 e LHR 111) compared to wild type animal (wt 005) of the same age (Figure11A).

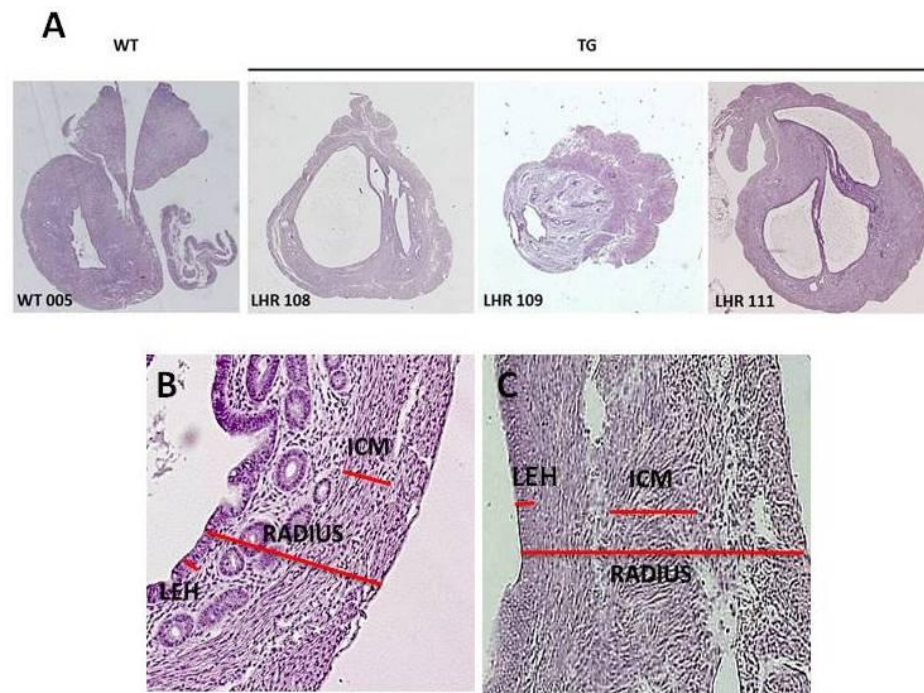


Figure 11: **A:** E/E staining was performed on uterus of 9-months old wt and TG-LHR mice. Magnification 1,6x. **B,C:** Representative samples of uterus showing Radius, ICM and LEH in 13-months old wt mouse (**B**) and 12-months old TG-LHR (**C**) mouse. Magnification10X.

For the subsequent assessment of the uterine morphometry of overexpressing LHR mice have been taken into account three parameters (GA Wood et al 2007): uterine radius, ICM (inner circular muscle) and LEH (luminal epithelium high) (Figure 11 B, C). It was observed an increase of the mean utrine radius in 6-12 months-old TG mice compared to wild-type mice (Figure 12); the same was observed for the thickness of the inner circular muscle (ICM). The luminal epithelial height (LEH) is similar in both wild type and TG-LHR mice. Moreover, transgenic and wt mice older than 12 months showed a similar radius thickness and LEH mean values. Instead, as concerning ICM, TG-LHR mice showed a mean thickness greater than wt animals (Figure 12).

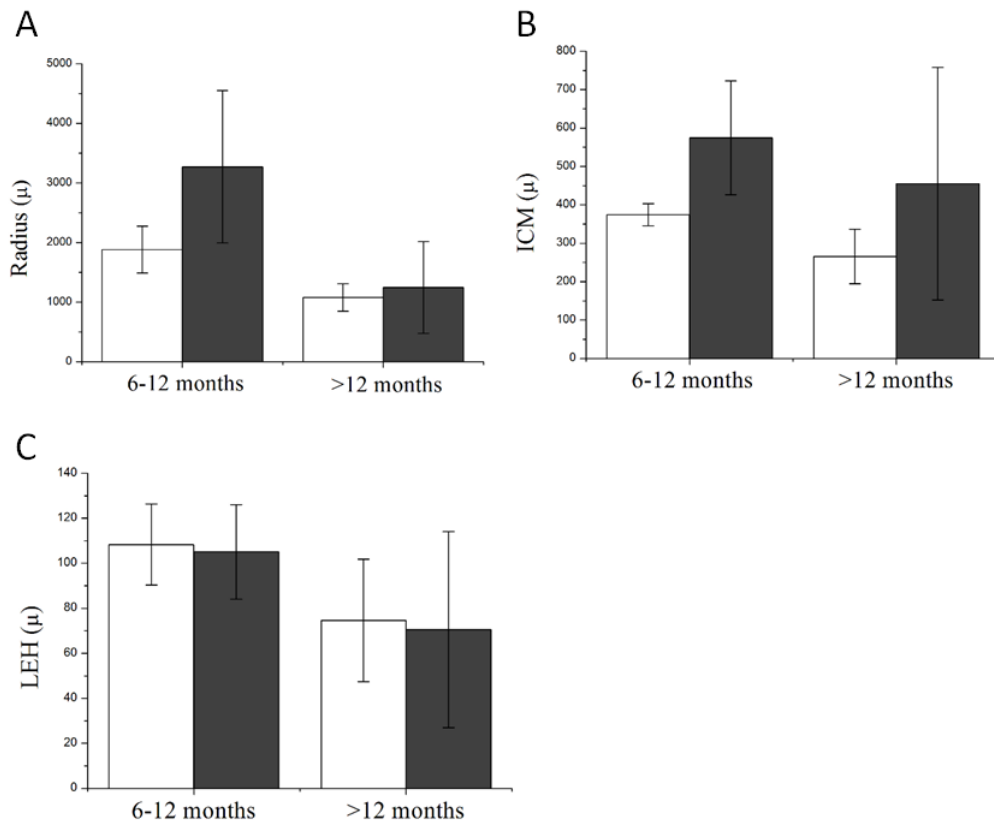


Figure 12: Mean of the Radius, ICM and LEH values of both wt (white bars) and TG-LHR (grey bars) mice. All the mice are divided into to groups: 6-12 months-old, and more than 12 months-old.

4.4 Immunohistochemical Characterization

Our next objective was to examine whether hLHR over-expression results in basement membrane alterations and myometrial invasion. Myometrial invasion is evaluated investigating the presence of α -smooth-muscle actin (α -sma) because it is frequently express in myofibroblasts (Daikoku T. et al 2008). In all the samples it was evaluated the expression of α -sma by immunihistochemical analisys with anti- α -sma antibody (as described in Materials and Methods).

In all the wild-type mice stained with α -sma (4 out of 4) the labeling was present only in the muscular cells (Figure 13A), while in TG-LHR mice samples (8 out of 8) the

labeling was present both in the muscular cells and in the glandular epithelium, probably indicative of transdifferentiation (Figure 13B).

We then performed Ki67 immunostaining in uterine sections of wild-type and TG-LHR mice in order to determine the proliferation status (Figure 13C and D).

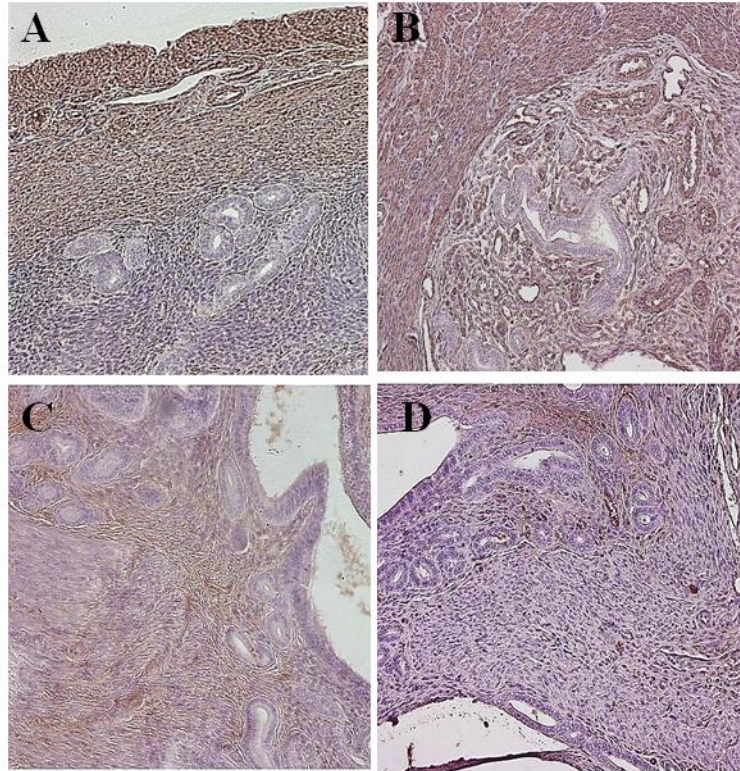


Figure 13: **A,B:** IHC was performed as described in Materials and Methods with anti α -sma antibody in wt mouse (**A**) and in TG-LHR mouse (**B**). Magnification 20X. **C,D:** IHC with anti Ki67 antibody. Negative sample for Ki67 staining (**C**) and positive sample for Ki67 staining (**D**). Magnification 20X.

The evaluation of Ki67 protein expression was performed taking into account the percentage of labeled nuclei in three different areas: the luminal epithelium, stromal cells and the endometrial glandular cells. We observed higher percentage of Ki67-positive cells in the stroma of 6-12 months-old transgenic mice compared to wild type animals, instead, a similar mean percentage of positive cells was observed between transgenic and wt mice older than 12 months. Moreover, Ki67 protein was found at

increased levels in the luminal epithelial cells of transgenic mice older than 12 months compared to wt mice of the same group of age (Table 2).

Age	WT	TG
6-12 months	Epithelial cells 3% Glandular cells 1% Stromal cells 18%	Epithelial cells 3% Glandular cells 5% Stromal cells 30%
> 12 months	Epithelial cells 3% Glandular cells 0% Stromal cells 20%	Epithelial cells 8% Glandular cells 1% Stromal cells 20%

Table 2: Mean percentage of positive cells to Ki67 staining in epithelial cells, glandular cells and stromal cells in both wt and TG mice. All the mice are divided into two groups of age: 6-12 months old and more than 12 months old.

All the animals sacrificed and used for morphometric and immunohistochemical characterization are reported and detailed in table 3.

MOUSE	AGE (mounth)	H & E	IHC C-MYC	IHC KI 67	IHC α -SMA
WT 5	9	X	-	Epithelial cells 0% Glandular cells 1% Stromal cells 30%	+ in uterine myocytes
WT 6	13	X	-	Epithelial cells 5% Glandular cells 0% Stromal cells 10%	+ in uterine myocytes
WT 8	18	X	-	Epithelial cells 5% Glandular cells 1% Stromal cells 30%	+ in uterine myocytes
TG LHR 108	9	X	+	Epithelial cells 0% Glandular cells 0% Stromal cells 20%	+ in uterine myocytes and glandular epithelium
TG LHR 109	9	X	+	Epithelial cells 0% Glandular cells 10% Stromal cells 40%	+ in uterine myocytes and glandular epithelium
TG LHR 111	9	X	+	Epithelial cells 0% Glandular cells 0% Stromal cells 30%	+ in uterine myocytes and glandular epithelium
TG LHR 101	12	X	+	Epithelial cells 0% Glandular cells 10% Stromal cells 30%	+ in uterine myocytes and glandular epithelium
TG LHR 103	12,5	X	+	Epithelial cells 10% Glandular cells 5% Stromal cells 40%	+ in uterine myocytes and glandular epithelium
TG LHR 105	17	X	+	+ in all the mass	+ in all the mass
TG LHR 207	13	X	+	Epithelial cells not evaluable Glandular cells 0% Stromal cells 20%	+ in uterine myocytes and glandular epithelium
TG LHR 211	16	X	+	Epithelial cells 5% Glandular cells 0% Stromal cells 5%	+ in uterine myocytes and glandular epithelium
TG LHR 123	17	X	+	50% mass cells; Epithelial cells 20% Glandular cells 0% Stromal cells 20%	+ in uterine myocytes and glandular epithelium
WT	23	X	-	Epithelial cells 0% Glandular cells 0% Stromal cells 20%	+ in uterine myocytes
WT 2	7			Epithelial cells 5% Glandular cells 0% Stromal cells 5%	
TG LHR 200 (6)	9		+	Epithelial cells 5% Glandular cells 0% Stromal cells 30%	

Table 3: Representation of all the mice used for morphological and immunohistochemical analysis. For each sample we performed hematoxilin and eosin (H & E) and immunohistochemical staining with anti-c-myc, anti-KI67 and anti- α -SMA antibodies.

4.5 Pathological findings in LHR-TG mice

In two 17-months-old mice (TG-LHR 105 and TG-LHR 123) we found a mass at the uterus level. In TG-LHR 105 mouse a huge mass it was localized in the lower abdomen (Figure 14 A, B, C), while in TG-LHR 123 mouse a smaller-sized mass was in the lower-third of the left uterine horn (Figure 14 A,B). Both the masses were taken and formalin-fixed and paraffin-embedded for immunohistological analysis. The tissue of the large-sized mass of TG-LHR 105 mouse was completely transformed and it is unrecognizable the typical uterine architecture (Figure 14D).

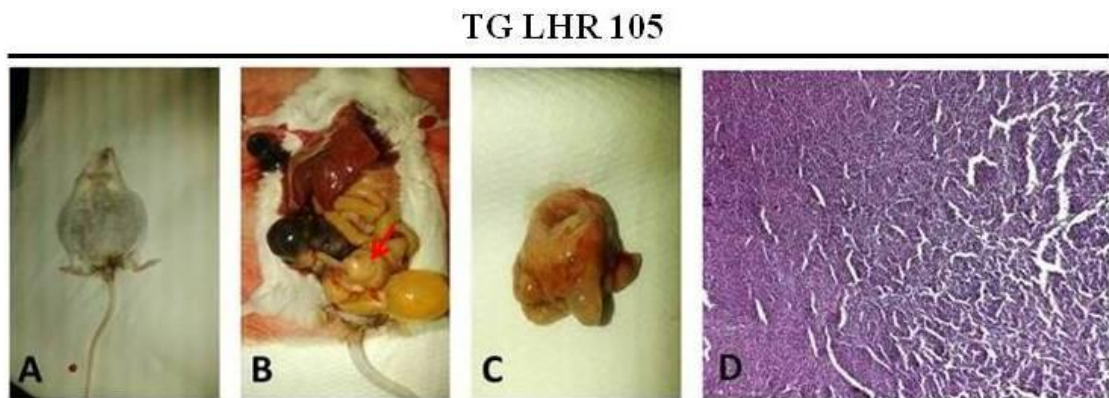


Figure 14: **A:** Macroscopic picture of TG-LHR 105 mouse, sacrificed at 17-months old. **B:** Tumor mass, indicates with the red arrow, at the moment of explant. **C:** Tumor mass. **D:** Mass stained with Ematoxilin and Eosin. Magnification 10x.

The mass of TG-LHR 123 mouse was small-sized and the tissue lost the normal uterine architecture, although the inner circular muscle layer was still visible (Figure 15 C, ICM is indicated with red arrow). All the elements that characterize the uterine tissue (inner circular muscle, uterine cavity, luminal epithelium) are maintained in the other healthy horn (Figure 15 D).

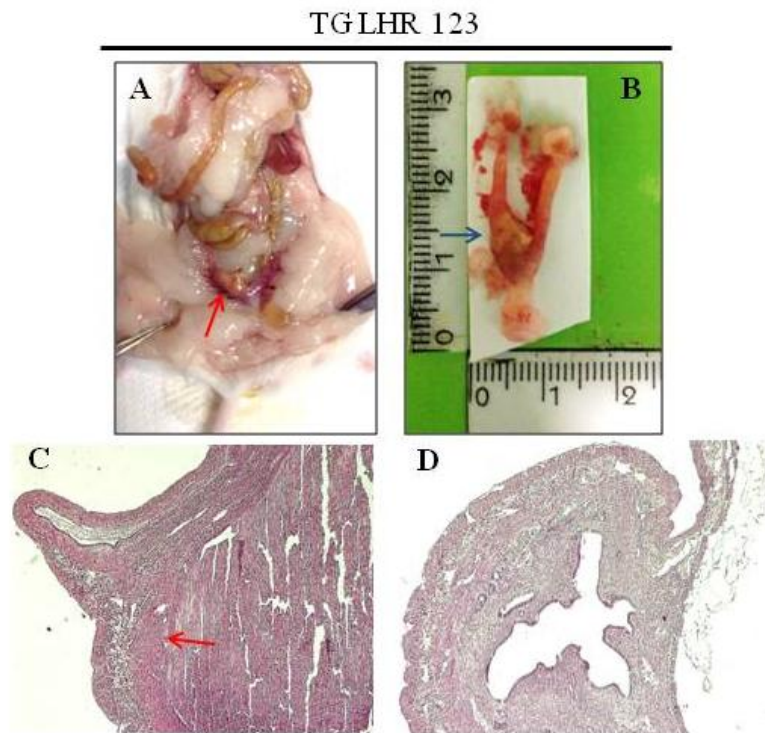


Figure 15: **A:** Macroscopic picture of TG-LHR 123 mouse during the explant; the red arrows indicates the uterus. **B:** Uterus explanted with mass in the lower-third of the left horn (blue arrow). **C:** Left horn with mass stained with E/E. Magnification 10x. **D:** Healthy right horn stained with E/E. Magnification 10x.

5. DISCUSSION

Published data highlight that the interaction between luteinizing hormone (LH) and its receptor (LHR) plays a role in the progression of endometrial cancer, especially in the acquisition of a more invasive potential. The increased ability of EC cells to undergo local invasion and metastatic spread, promoted by the binding of LH to its receptor, was also demonstrated by our group in an orthotopic/menopausal mouse model (Pillozzi S et al 2013). In order to evaluate the effect of LHR over-expression and to assess if it could be capable *per se* of inducing the development of an EC, we produced a transgenic mouse model over-expressing LHR in female reproductive tracts. The real time PCR and the immunohistochemistry analysis confirmed the presence of the transgene in the uterus and ovary in both the two lines of transgenic mice (LHR-100 and LHR-200). Studying the expression of hLHR mRNA in TG-LHR mice it emerged that in uterus and liver the transgene is expressed at high levels and the different expression level between transgenic and wt animals it is statistically significant. The hepatic ectopic overexpression of the transgene it is probably due to the insertion site of the transgene or to epigenetic causes. Analysis to determine the transgene copy number inserted in transgenic animals are ongoing. Further analysis were performed to characterize the uterine morphometry taking into account the uterine radius, inner circular muscle (ICM) and luminal epithelial height (LEH). Radius and ICM were increased in 6-12 months-old transgenic mice compared to wt animals of the same age, whereas no differences were observed in LEH. On the contrary, radius thickness and LEH showed a similar mean values in both transgenic and wild type mice older than 12 months. Instead, as concerning ICM, TG-LHR mice showed a mean thickness greater than wt animals. To

deepen this topic, we investigated cell proliferation evaluating Ki67 staining in epithelial, glandular and stromal cells of the uterine mucosa. From such analysis, it emerged that 6-12 months-old TG mice showed higher proliferation rate compared to wt mice, whereas in mice older than 12 months the proliferation rate was similar both in wt and TG mice. Moreover, old mice over-expressing LHR showed an increased proliferation rate in epithelial luminal cells compared to wt. We then examined whether LHR over-expression might affect basement membrane alterations and myometrial invasion by investigating the presence of α -smooth-muscle actin (α -sma) that is known to be frequently expressed in myofibroblasts (Daikoku T. et al 2008). Differences were observed between transgenic and wt mice of both age groups. In particular, α -sma was expressed only in muscular cells of wt mice, whereas in all TG mice it was expressed not only in the myocytes but also in the glandular epithelium, probably indicating transdifferentiation.

From the histological analysis it emerged that two 17-months old TG mice developed tumor masses. Interestingly, in LHR-105 TG mouse a huge masses occupying the entire abdomen was observed, while in TG LHR-123 the mass was localized in the lower-third of the left uterine horn. Although these results were observed in a small subset of animals, they are quite interesting since might suggest a potential causative role of LHR in endometrial cancer pathogenesis, besides its role in tumor invasion and metastatic potential (Pillozzi S et al 2013). Considering LHR ability to regulate PKA signaling (Dabizzi S et al 2003), such scenario could be related to the modulatory effects that LHR exerts on intracellular signaling pathways which control cell proliferation, survival and invasiveness. This topic deserves further attention and it is worth investigating in the future. Additional microarray analyses are ongoing to better investigate the pattern of genes involved in the developing of the two masses, to deepen this topic and better characterize LHR transgenic animals.

The effect of the over-expression of LHR will be further evaluate on the susceptibility to carcinogenic agents. The use of such model allowed us to conclude that TG LHR mice may represent a useful model for the study of endometrial cancer *in vivo* and in particular to assess therapeutic intervention and pharmacological tests.

CHAPTER 3

In last years many data have been collected about the *in vitro* and *in vivo* role of luteinizing hormone receptor (LHR) that tend to configure the LH/LHR axis as a progression factor in EC, contributing to regulate cell invasion and angiogenesis, and hence ultimately leading to metastatic spread. In this part of the thesis we evaluated the expression levels of LHR gene in a case series of EC. In addition, we also evaluated in the case series the mRNA levels of two ion channels (hERG1 and KCNA7), known to be dysregulated in EC.

1. INTRODUCTION

1.1 EPIDEMIOLOGY OF ENDOMETRIAL CANCER

Endometrial cancer (EC) is nowadays the most common gynecologic malignancy and the most frequent among infiltrating tumor of the female genital tract, the sixth most common cancer in women worldwide (fourteenth most common cancer overall), with 319.000 new cases and 76.000 deaths per year (Ferlay J et al. 2012; Sartori E. et al 2010; Matias-Guiu X. et al 2001). Approximately 75% of the cases are related to the corpus uteri and 15-20% of these have relapse and are unresponsive to systemic therapy (Amant F. et al. 2005). In the Europe about 99.000 new cases of EC and 23700 deaths were reported in 2012. Concerned Italy, it is at the eighteenth position in Europe whit 8471 new cases with 1955 death every year (Ferlay J et al. 2012) (Figure1).

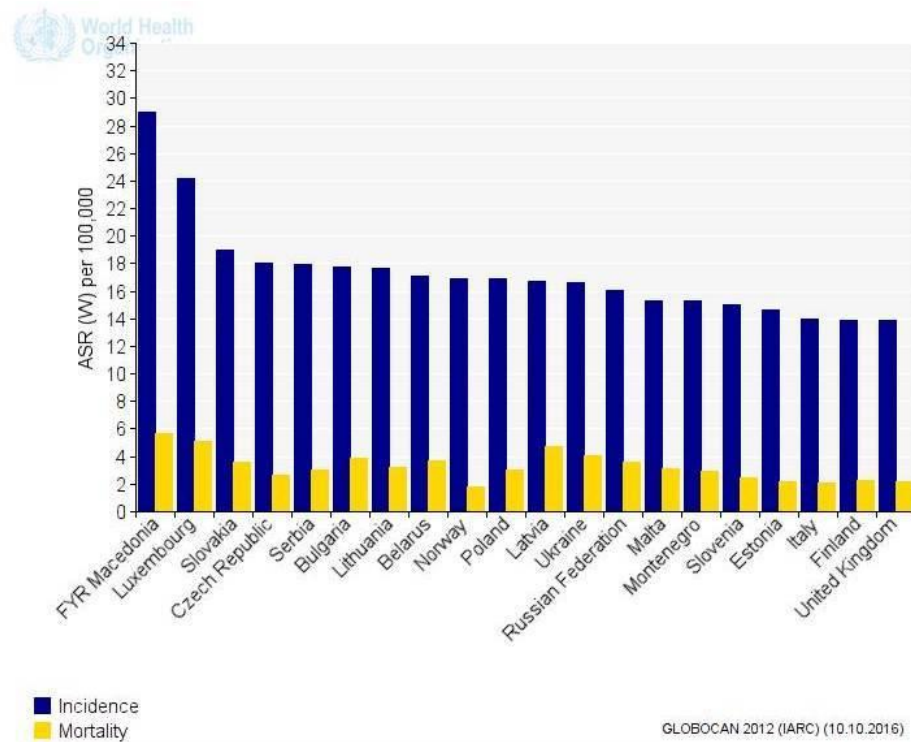


Figure 1: Bar chart representing the incidence and the mortality of EC in the 20 European countries with highest incidence of Endometrial Carcinoma (Globocan 2012).

The analysis of EC incidence trends showed an increasing during '60s of the last century with a peak during the 70s and than a subsequent stabilization. The incident trend showed an increase in postmenopausal age and in particularly in industrialized countries, rather than in developing countries (Cook SL et al., 2006). There are distinct patterns in the age-specific trends of EC. A general profile emerged of increasing risk in postmenopausal women (ages >55 years), and decreasing or stable trends in premenopausal and perimenopausal women (ages 30-54 years), particularly in Northern and Western countries. The most consistent declines in these regions were observed in women ages 45 to 54 years (Bray F. et al., 2005). The incidence of EC among white women it is higher compared with African-American women, while five-year survival was poorer for African-American women, even for patients with the more favorable Stage I adenocarcinoma who were treated surgically (Hicks ML et al 1998). In Tuscany

the risk for a woman (from 0 to 84 years) to develop EC is about 2% (1 in 50 women), whereas the mortality rate is approximately 0,1% (1 in 736 women).

1.2 CLASSIFICATION

1.2.1 FIGO classification

The first classification of Endometrial Cancer was formulated by F.I.G.O (International Federation of Gynecology and Obstetrics) in 1971 and consists in classification of EC in stages, based on clinical data, analysis of endometrial biopsy or measurement of the uterine cavity. This classification has three stages of EC: Stage 0 includes *in situ* carcinomas, Stage I includes tumors confined to corpus uteri, Stage II includes tumors that invades cervix. This first classification was incomplete because do not consider pathological parameters such as histological grading, myometrial infiltration, peritoneal histology and lymph nodal diffusion. In 1988 it was introduced by F.I.G.O. a new classification based on pathological factors. A revised version was introduced in 2009, with a rationale to further improve the prognostic performance of surgical staging. Main changes for the FIGO 2009 system include: noninvasive tumors and tumors with <50% myometrial invasion are combined; cervical glandular involvement does not affect staging; peritoneal cytology does not affect staging; tumors with lymph node metastasis are subdivided to stages IIIC1 and IIIC2 (Table 1) (Haltia UM et al 2014; Amant F et al 2012).

FIGO Stage	
I ^a	Tumor confined to the corpus uteri
IA ^a	No or less than half myometrial invasion
IB ^a	Invasion equal to or more than half of the myometrium
II ^a	Tumor invades cervical stroma, but does not extend beyond the uterus ^b
III ^a	Local and/or regional spread of the tumor
IIIA ^a	Tumor invades the serosa of the corpus uteri and/or adnexae ^c
IIIB ^a	Vaginal involvement and/or parametrial involvement ^c
IIIC ^a	Metastases to pelvic and/or para-aortic lymph nodes ^c
	IIIC1 ^a : Positive pelvic nodes
	IIIC2 ^a : Positive para-aortic nodes with or without positive pelvic lymph nodes
IV ^a	Tumor invades bladder and/or bowel mucosa, and/or distant metastases
IVA ^a	Tumor invasion of bladder and/or bowel mucosa
IVB ^a	Distant metastasis, including intra-abdominal metastases and/or inguinal nodes)
^a	Either G1, G2, or G3.
^b	Endocervical glandular involvement only should be considered as Stage I and no longer as Stage II.
^c	Positive cytology has to be reported separately without changing the stage.

Table 1: Stages of endometrial carcinoma (Amant F et al 2012).

Another classification use the TNM staging system, in which are taken into account three parameters: the size of the primary tumor (T); regional lymph nodes involvement (N); presence of distant matastasis. Comparison of the F.I.G.O. with the TNM classification is given in Table 2.

FIGO Stage	Union for International Cancer Control (UICC)		
	T (tumor)	N (lymph nodes)	M (metastasis)
I	T1	N0	M0
IA	T1a	N0	M0
IB	T1b	N0	M0
II	T2	N0	M0
III	T3	N0–N1	M0
IIIA	T3a	N0	M0
IIIB	T3b	N0	M0
IIIC1	T1–T3	N1	M0
IIIC2	T1–T3	N1	M0
IVA	T4	Any N	M0
IVB	Any T	Any N	M1

Table 2: Endometrial carcinoma: FIGO staging compared with the TNM classification (Amant F et al 2012).

1.2.2 Grading

Furthermore for each stage it is important to associate a Grading system, which represent the differentiation rate of the tumor.

- **G1:** Well differentiated (Low grade)
- **G2:** Moderately differentiated (Intermediate grade)
- **G3:** Poorly differentiated (High grade)

Cases of EC should be grouped with regard to the degree of differentiation of the adenocarcinoma as follows:

- G1: <5% of a nonsquamous or nonmorular solid growth pattern
- G2: 6%-50% of a nonsquamous or nonmorular solid growth pattern
- G3: >50% of a nonsquamous or nonmorular solid growth pattern

1.2.3 Histopathologic Classification

Two major types of EC are distinguished on the bases of histological features:

Type I: commonly referred to as the endometrioid type it is estrogen-dependent and develops through the hyperplasia-carcinoma sequence. These tumors comprises 80% of all endometrial cancers and arises in relatively younger pre- and post-menopausal women. They usually are low grade with an endometrioid morphology, and they are characterized by a favorable prognosis. Histologically, these tumors can be adenocarcinoma with or without squamous differentiation and often are well differentiated. Furthermore, epidemiological evidence suggests that the multistep carcinogenic process of Type I endometrial tumors begins with simple endometrial hyperplasia, progresses to complex atypia hyperplasia, and then develops into the precursor lesion, endometrial intraepithelial neoplasia (EIN) (Felix AS et al 2010).

Type II: Type II EC, or non-endometrioid tumors, encompasses the remaining 10–20% of sporadic endometrial tumors. The two histologies of this subtype are uterine papillary serous carcinoma and clear-cell carcinoma (Felix AS et al 2010). These tumors arises in relatively older women and are not usually preceded by a history of unopposed estrogen exposure, but rather from a background of atrophic endometrium. Type II tumors have an aggressive clinical course, a greater propensity for early spreading, and a worse prognosis than the more common endometrioid adenocarcinomas (Doll A et al 2008). While the incidence of Type II tumors is low compared to Type I, excess mortality is associated with Type II. The main features of Type I and Type II EC are summarized in Table 3.

Characteristic	Type I	Type II
Unopposed oestrogen	Yes	No
Background endometrium	Hyperplastic	Atrophic
Morphology	Endometrioid	Serous, clear cell
Genetic abnormalities	MSI, PTEN, <i>K-ras</i> , β -catenin	p53, <i>HER2/neu</i>

Table 3: Clinico-pathological characteristics and genetic abnormalities in Type 1 and Type 2 endometrial carcinomas (Ryan AJ et al 2005).

The prognostic value of these two main subgroups are limited because it does not explain the causes of 20% recurrences in the Type I EC and 50% recurrences in Type II EC (Rose PG, 1996).

1.3 RISK FACTORS FOR EC DEVELOPMENT

Although the etiology is not completely clear, the endometrial carcinoma is associated with many risk factors:

Obesity: large body mass in general, and obesity in particular, has been linked to an increased risk of endometrial cancer in many studies. Some studies also suggest that the association between large body mass and endometrial cancer is stronger or more consistent in post-menopausal women (Tornberg SA et al 1994). An association with obesity is biologically plausible. Post-menopausal obese women are known to have higher endogenous oestrogens than lean women⁶⁵ due to the aromatization of androstenedione in adipose tissue (Austin H et al 1991; Purdie DM 2001).

Estrogens: The predominant theory describing the relationship between endogenous steroid hormones and endometrial cancer risk is known as the unopposed estrogen hypothesis. This hypothesis proposes that endometrial cancer risk is increased in women who have high plasma bioavailable estrogens and/or low plasma progesterone, so that mitogenic effects of estrogens are insufficiently counterbalanced by progesterone

(Key TJ et al 1998). This theory originated from at least two important observations: (a) increased endometrial proliferation rates during the follicular phase of the menstrual cycle, during which progestin levels are low, whereas E₂ levels are at normal premenopausal concentrations; (b) increased endometrial cancer risk among women using exogenous estrogens without progestins (Hormonal contraception and postmenopausal hormonal therapy, 1999; Kaaks R et al 2002). The use of the combined oral contraceptive pill (estrogen plus progestogen) reduces the risk of endometrial cancer has been reported consistently and the protective effect of the combined oral contraceptive pill does not depend on the dose of the progestogen (Parslov M et al 2000; Purdie DM 2001; Weiderpass E et al 1999). Long-term use of combined oral contraceptives seems to reduce the risk further, and the protective effect lasts for 20 or more years after discontinuation (Weiderpass E et al 1999).

Tamoxifen therapy: tamoxifen has an anti-estrogenic effect and is often used as adjuvant therapy for women with breast cancer. Results from clinical trials in women with breast cancer have highlighted a potential increase in endometrial cancer resulting from use of these anti-estrogens (Mignotte H et al 1998). The risk has also been seen to increase with increasing duration of treatment with tamoxifen and increasing cumulative dose.

Nulliparity/infertility: nulliparity could be a manifestation of infertility, which, in turn, has been separately identified as a risk factor for endometrial cancer. Infertility is difficult to measure retrospectively with accuracy, however, and therefore the risk associated with it is difficult to quantify. Numerous surrogate measures have been used, for example, married nulliparous women have been found to be at greater risk than unmarried nulliparous women (Parazzini F et al 1991).

Diabetes: Diabetes mellitus is another condition long known to be associated with endometrial cancer (Weiderpass E et al 2000; Elwood JM et al 1997). One possible

explanation is that these conditions are simply markers of obesity. It has been suggested that the increased risk associated with diabetes may be restricted to obese or overweight women (Weiderpass E et al 2000).

1.4 MOLECULAR BIOLOGY OF EC

EC is characterized by a variety of genetic alterations, the most frequent of which is to the *PTEN* gene. The genes code for proteins inhibiting tumor growth. *PTEN* encodes a protein (phosphatase and tensin homolog, PTEN) with tyrosine kinase function and behaves as a tumor suppressor gene. PTEN has been reported to be altered in up to 83% of endometrioid carcinomas and 55% of precancerous lesions (Mutter GL 2001). PTEN inactivation is caused by mutations that lead to a loss of expression and, to a lesser extent, by a loss of heterozygosity. Loss or altered PTEN expression results in aberrant cell growth and apoptotic escape. Loss of PTEN is furthermore probably an early event in endometrial tumorigenesis. Its expression is highest in an estrogen-rich environment; in contrast, progesterone promotes involution of PTEN-mutated endometrial cells (Kim YB et al 1997; Tsikouras P et al 2013).

Mutations in *PIK3CA* may contribute to the alteration of the phosphatidylinositol 3 kinase (PI3K)/AKT signaling pathway in EC (Yeramian A et al 2012). A high frequency of mutations in the *PIK3CA* gene has been reported in EC. *PIK3CA* mutations occur in 24–39% of the cases, and frequently coexist with PTEN mutations. *PIK3CA* mutations have been associated with adverse prognostic factors such as high-grade and myometrial invasion (Oda K et al 2005). The most common genetic alteration in type 2 serous carcinomas is in p53, the tumor suppressor gene. Mutations in p53 are present in about 90% of serous carcinomas (Lax S et al 2004). The exact mechanism behind the cause of this mutation is still unclear but it is postulated that mutation in one

allele occurs early during the development of serous carcinoma, and loss of the second normal allele occurs late in the progression to carcinoma. Other genetic alterations in endometrioid EC include microsatellite instability (MSI) and specific mutations of K-ras and β -catenin genes. MSI and K-ras mutations have been reported in about 20% of endometrioid cancer (Kandoth C et al 2013; Yeramian A et al 2012). Frequent genetic alterations in type 2 ECs are inactivation of p16 and overexpression of HER-2/neu (Konecny GE et al 2009). P16 inactivation was found in 45% of serous carcinomas and some clear cell cancers. The p16 tumor suppressor gene is located on chromosome 9p21 and encodes for a cell cycle regulatory protein. Inactivation of p16 leads to uncontrolled cell growth. HER-2/neu overexpression has been associated with a metastatic phenotype and poor survival in type 2 EC (Konecny GE et al 2009).

The recent mapping of the genomic landscape of serous and endometrioid endometrial carcinomas has resulted in the first comprehensive molecular classification of these tumors and has distinguished four molecular subgroups: a POLE/ultramutated subgroup, a hypermutated/microsatellite unstable subgroup, a copy number low/microsatellite stable subgroup, and a copy number high subgroup. This molecular classification may ultimately serve to refine the diagnosis and treatment of women with endometrioid and serous endometrial tumors (Le Gallo M et al 2015).

1.5 LHR AND ENDOMETRIAL CANCER

Endometrial cancer occurs most commonly in postmenopausal women and is therefore coincidentally associated with elevated plasma LH levels (Nagamani et al, 1993). Studies strongly suggest that high postmenopausal levels of LH are stimulating endometrial cancer cell growth but not via the classical LH receptor-cAMP pathway (Davies S et al, 2000). LH receptor gene expresses in endometrium normal tissue at very low levels, while an increased expressions of LHR has been found in endometrial

cancerous tissues (Ji Q et al, 2002). The activation of LHR in endometrial cancer cells is established that promotes cancer local invasion metastatic progression and the capability of invading in response to LH addition was dependent on PKA activation (Dabizzi S et al, 2003; Noci I et al 2008) The mechanism for this involves LH binding to its receptor and inducing the activation of PKA, which in turn induces a functional activation of beta 1 integrin receptors and the subsequent secretion of active matrix metalloproteinase-2 ending in the triggering of cell invasiveness (Dabizzi S et al, 2003). The increased ability of EC cells to undergo local invasion and metastatic spread, promoted by the binding of LH to its receptor, was also demonstrated by our group in an orthotopic/menopausal mouse model (Pillozzi S et al 2013). The model consisted in an orthotopic xenograft of Hec1A cells into immunodeficient mice treated with recombinant LH, to assure high levels of LH. In particular, it was observed that tumors arising from Hec1A-LHR cells injection displayed a higher local invasion and a higher number of distant metastases, mainly in the lung, compared to tumors obtained from the injection of Hec1A cells. LH withdrawal strongly inhibited local and distant metastatic spread of tumors, especially those arising from Hec1A-LHR cells (Pillozzi S et al 2013). In a recent paper have been described high levels of expression of LHR at both mRNA and protein levels in a patient with EC who experienced early relapses, even though she was defined as low risk according to the current guidelines. For this reason it may be possible to sustain that there is a relationship between high LHR expression and biological aggressiveness of EC (Noci et al, 2016).

1.6 hERG1 CHANNEL AND ENDOMETRIAL CANCER

EC is a heterogeneous disease and novel biomarkers are urgently needed in order to better stratifying EC patients and ensuring the best treatment options. The first paper demonstrating the expression of hERG1 potassium channels in human primary cancers

was conducted on EC samples (Cherubini A et al 2000). In such paper it was demonstrated that hERG1 mRNA can be detected in human tissues by end-point RT-PCR as well as by immunohistochemistry and is more frequently expressed in human neoplastic tissues compared to normal endometrium and hyperplastic lesions (Cherubini A et al 2000). Furthermore, patch clamp analysis indicated that functional hERG1 proteins are expressed on the cell surface of EC cells. This paper opened the way for further investigation of hERG1 expression in clinical samples, although the analysis was carried out on a small group of EC patients.

2. MATERIALS AND METHODS

2.1 Description of the prospective cohort

For this study, we enrolled a cohort of 123 patients with pathologically confirmed endometrial cancer treated at the AOUC Careggi Hospital from 19 March 2013. Samples were collected after obtaining an informed written consent from all the patients at the moment of surgery in RNA later (Sigma-Aldrich), in order to preserve RNA integrity. After the collection the tumor samples were conserved at -80°C . The treatment protocol was developed according to ITT (Istituto Toscano Tumori) guidelines and international guidelines. A database has been created in order to collect the clinical and pathological parameters such as tumor histotype, grade of differentiation, FIGO stage, myometrial invasion, presence or absence of menstruation. Not all clinical pathological parameters were available for all patients involved in this study. Patients are ranged from 39 to 92 years, with a median age of 65 years. Post-menopausal women were hundred and twelve (89%) at the moment of the surgery. EC patients enrolled in the study were divided in two subgroups based on histological type. Hundred and eleven (90%) patients had an endometrioid tumor histotype, whereas twelve (10%) patient had a non-endometrioid histotype (Figure 7).

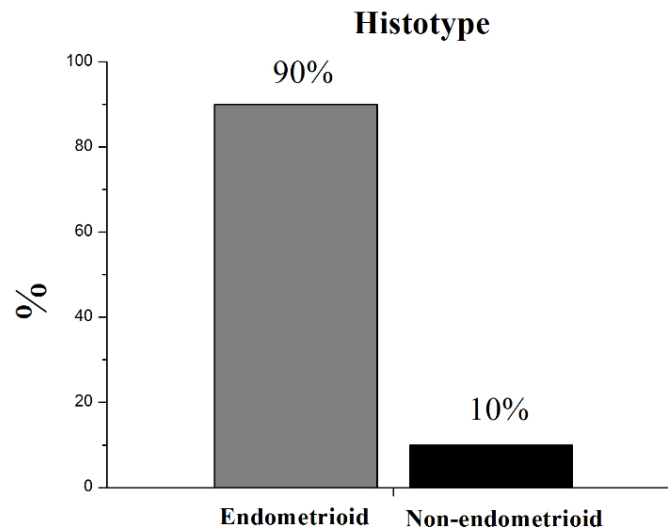


Figure 7: Percentage of patients belonging to Endometrioid and Non endometrioid.

The case series was also divided according to the FIGO classification. One hundred-three patients (86%) belong to the Stage I (Ia and Ib), seven (6%) belong to the Stage II and nine (8%) to the Stage III (IIIa, IIIb and IIIc) (Figure 8).

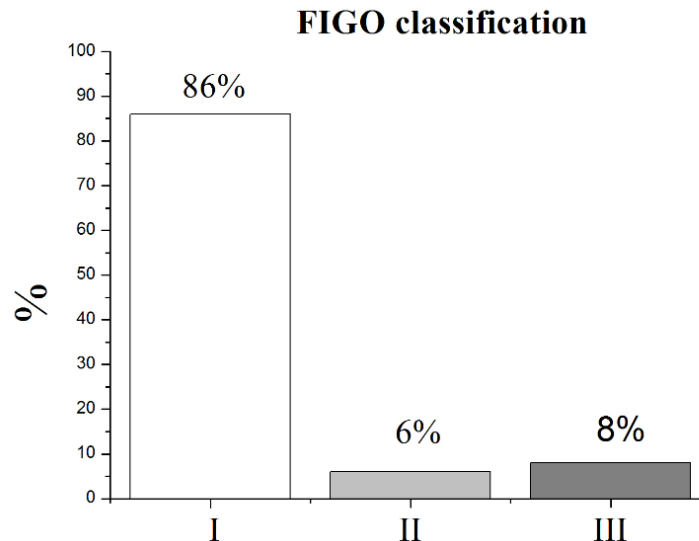


Figure 8: Distribution of patients based on FIGO staging.

Among 123 patients, the most represented histological grade was the G2, with fiftyeight patients (47%). In thirtyfive patients (29%) the tumor was poorly differentiated (G1), whereas in thirty patients (24%) the tumor was well differentiated (G3) (Figure 9).

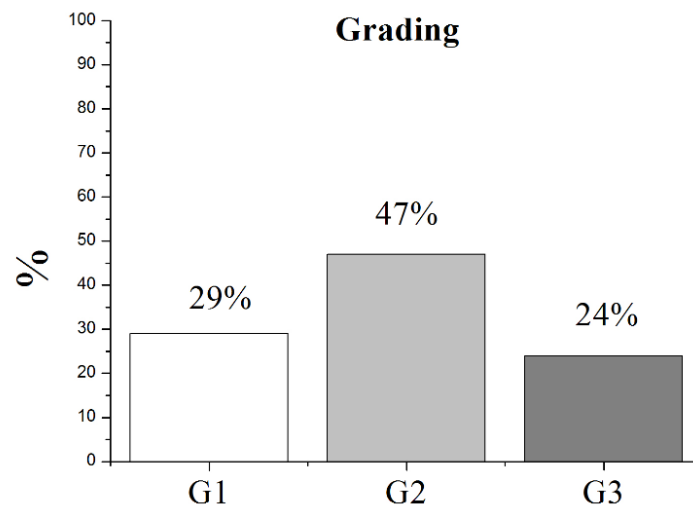


Figure 9: Patients distribution based on histological grade.

Traditionally, the percentage of myometrial invasion, categorized as $<50\%$ or $>50\%$, is one of the parameters used in the determination of the need for adjuvant radiotherapy. In sixty-eight (59%) cases of EC patients the infiltration was less than $1/2$ myometrial depth, whereas in forty-eight (41%) it was greater than $1/2$ myometrial depth (Figure 10).

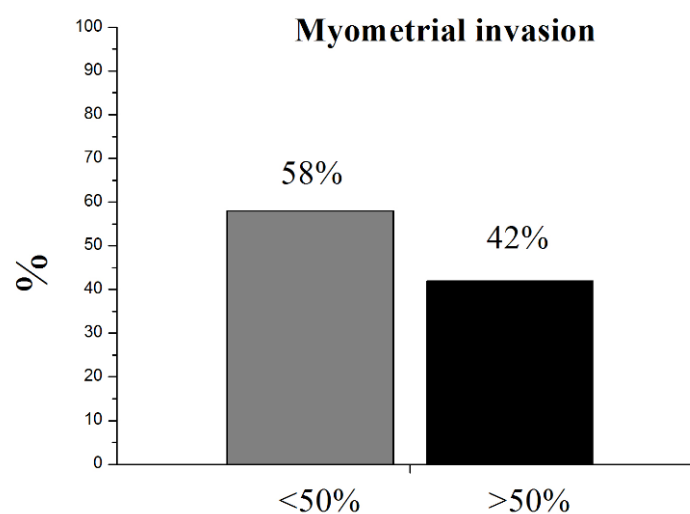


Figure 10: Patients distribution on the basis of myometrial infiltration: $<50\%$ myometrial infiltration vs $>50\%$ myometrial infiltration.

All the patients enrolled in this study were divided in two groups based on clinical and pathological parameters:

- Low-Risk EC subgroup: Endometrioid EC, G1 or G2 grading, <50% myometrial invasion (Stage Ia).
- High-Risk EC subgroup: Endometrioid EC+G3 grading and/or >50% myometrial invasion (Stage \geq Ib). Non endometrioid EC.

Sixty-nine patients (55%) belong to Low-Risk subgroup, whereas fifty-seven (45%) patients belong to High-Risk subgroup (Figure 11).

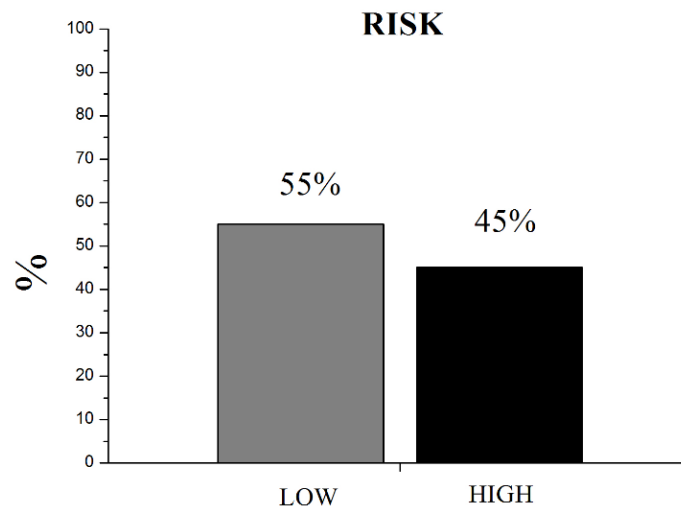


Figure 11: On the bases of histological type, histologic grade and myometrial invasion, patients are divided in two subgroups: Low-risk and High-risk.

2.2 RNA extraction and reverse transcription

Fresh endometrial cancer specimens were dipped in TRIzol Reagent (Invitrogen) and homogenized to isolate total RNA according to manufacturer's protocol. The cDNA was synthesized from 1 μ g of total RNA using 200U/ μ L of SuperScript II Reverse Transcriptase (Invitrogen), adding for each sample 500 μ mol/L of deoxyribonucleotide triphosphate (dNTP) (Invitrogen) and 15 ng/ μ L of random primers (Invitrogen).

The reaction starts preparing a mix with:

- 1 µg of RNA
- 2 µl of Random Primers (diluted 1:20 from the stock solution)
- 1 µl of dNTPs (10mM)
- ddH₂O till a final volume of 12 µl

The mix was incubated in a thermal cycler at 65°C for five minutes, and then immediately placed on ice for five minute. Then, 4 µl of Buffer 5X First Strand (Invitrogen) and 3 µl of ddH₂O were added to the tube. The mix was heated at 25 °C for 2 min and than added with the SuperScript II. The mix was than incubated as follows: a first step at 25°C for 10 min followed by 50 min at 42°C and a final step at 70°C for 15 min.

2.3 Real Time PCR

hLHR, KCNH2 and KCNA7 expression levels were evaluated by real-time quantitative PCR (qPCR) using 1 µL of cDNA. The expression levels of genes were normalised to the levels of GAPDH housekeeping gene. SYBR green fluorescent dye were used to monitor the DNA synthesis. The primers pairs for LHR, KCNH2, KCNA7 and GAPDH were designed to spanning intron/exon boundaries. All the Primers used for the clinical study are listed in table 4.

GAPDH L	5' GCTCTCGCTCCTCCTGTT 3'
GAPDH R	5' ACGACCAAATCCGTTGACTC 3'
LH/R 2/3 UP	5' TGCCTACCTCCCTGTCAAAG 3'
LH/R 2/3 DN	5' TTGAGGAGGTTGTCAAAGG 3'
KCNA7_AL	5' CTTCAATGACCCGTTCTTCGT 3'
KCNA7_AR	5' AAGTAGGGAAGGATAGCCACAA 3'
KCNH2-AF1-L	5' ACGTCTCTCCCAACACCAAC 3'
KCNH2-AF1-R	5' GAGTACAGCCGCTGGATGAT 3'

Table 4: Primers sequences

All the primers were used at final concentration of 300 nM. PCR program start with an incubation at 95 °C for 10 min, followed by 40 cycles of amplification: denaturation at 95 °C for 15 s and annealing–extension step at 60 °C for 1 min. The relative quantification of hERG1 expression levels was performed by the Δ Ct method. Each reaction was performed in triplicate. Melting curve analysis of the amplicons were performed to exclude the amplification of a specific products or primer-dimer artefacts. Relative expression values of LHR and KCNA7 were normalized for the expression value of Hec1A cells (low expression cell line). Expression values of KCNA7 gene was normalized for the expression value of MDA-MB cells (low expression cell line).

2.4 Statistical analysis

For the statistical analysis clinical-pathological parameters were categorized as follows: age at the diagnosis; histology= endometrioid vs non endometrioid; differentiation grade= low, medium, high; FIGO= I, II, III and IV; risk= low vs high; myometrial invasion= less than 50% vs greater than 50%; postmenopause; body mass index (BMI). The associations between the values of expression LHR, KCNH2 and

KCNA7 and clinical-pathological parameters were assessed applying the Spearman correlation test. $P < 0,05$ was considered statistically significant. For the follow-up analysis the event free survival time (EFS) of patients was defined as the interval between the intervention and the relapse of the disease. Univariate analyses of event free survival time were calculated according to the Kaplan-Meier method. Comparisons between survival curves were performed using the long-rank test. For the EFS analysis LHR, KCNH2 and KCNA7 genes were categorized as 0/1 respect to their level of expression, lesser or greater than the median value. The statistical analysis were performed in collaboration of Dott. L. Tofani (University of Florence).

3. AIMS OF THE STUDY

The aims of the present study was to assess the expression levels of a gonadotropin receptor, such as LH receptor, and two K⁺ ion channels (hERG1 and KCNA7) in a prospective cohort of primary EC samples provided by Azienda Ospedaliero-Universitaria, Careggi, Florence. Furthermore we designed the present study in order to assess and validate the correlation between the expression levels of the three genes and clinical-pathological data and among the expression. The final goal will be to unravel the predictive value of LHR, KCNH2 and KCNA7 expressions levels to better stratify EC patients.

4. RESULTS

Endometrial cancer is a heterogeneous disease and novel biomarkers are urgently needed. This will allow better stratifying EC patients and ensuring the best treatment options. In recent years it was studied the role of LHR in endometrial carcinoma. In particular it seems to promote local invasion and metastatic spread (Pillozzi S et al 2013). Ion channels are involved in tumor progression in many types of cancer, in particular KCNH2 was demonstrated to be over-expressed in endometrial cancer (Cherubini A et al 2000). Moreover, preliminary data coming from a microarray analysis identified a number of ion channels with an expression profiles that may suggest their involvement in EC. We focused on LHR, KCNH2 and KCNA7 (potassium voltage-gated channel, shaker-related subfamily, member 7) genes because they seem to be the most dysregulated genes.

4.1 Evaluation of expression levels of LHR, KCNH2 and KCNA7

We evaluated the gene expression levels of LHR, KCNH2 and KCN7 in endometrial cancer by real time PCR. LH receptor expression level was analyzed on all the 123 EC samples collected at the AOUC Careggi Hospital, Florence. The GAPDH housekeeping gene was amplified as a control (as describe in Materials and Methods). Overall, we found a strong variability in LHR expression level in the specimens analyzed, with a range between 0,26 and 5722973, and a median value of 73,78 (Figure 12).

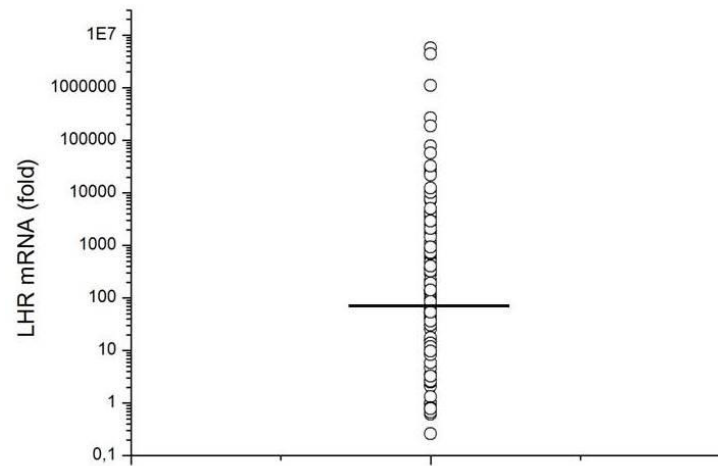


Figure 12: Distribution of LHR mRNA expression values. The black line represent the median.

The EC patients were evaluated also for the expression of KCNH2 and KCNA7 genes, because these ion channels are known to be dysregulated in several types of tumors. KCNH2 expression level ranged from 0,09 to 70077, with a median value of 76,11 (Figure 13).

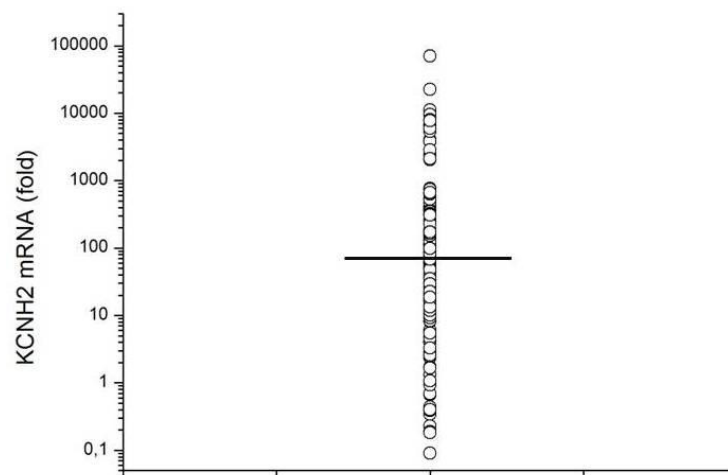


Figure 13: Distribution of KCNH2 mRNA expression values. The black line represent the median.

Real time PCR analysis showed that KCNA7 expression was extremely heterogeneous in the cohort of patients, with a range between 0,01 and 4555 and a median value of 0,7 (Figure 14).

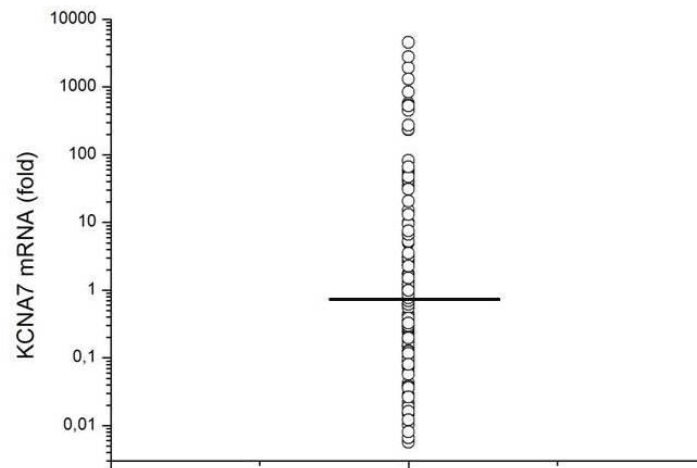


Figure 14: Distribution of KCNH2 mRNA expression values. The black line represent the median.

4.2 Statistical analysis

LHR, KCNH2 and KCNA7 expression values were used to perform statistical analysis applying the Spearman Correlation Test (table 5). We observed that the expression of LHR is statistically associated with KCNH2 ($p=0,0064$) and KCNA7 ($p<0,001$). Furthermore, the expression of KCNH2 with KCNA7 is statistically associated ($p<0,001$).

	Spearman index	P value
LHR/KCNH2	0,245	0,0064
LHR/KCNA7	0,435	<0,0001
KCNH2/KCNA7	0,561	<0,0001

Table 5: Table representing the associations between LHR, KCNH2 and KCNA7 gene expression calculated with Spearman method and P values associated to the Spearman index.

We then evaluated whether the expression of LHR, KCNH2 and KCNA7 were associated with clinical-pathological parameters. Statistically significant correlations were found between LHR mRNA high expression and low risk endometrial cancer ($p=0,025$) and between LHR overexpression and infiltration less than 50% myometrial depth. Statistically significant associations were found also between KCNH2 high expression and low FIGO stages ($p=0,01$), low risk endometrial cancer ($p=0,019$) and infiltration less than 50% myometrial depth ($p=0,036$). Moreover, KCNA7 overexpression was statistically associated with low risk endometrial cancer ($p=0,018$), invasion less than 50% myometrial depth ($p=0,018$) and postmenopause ($p=0,048$) (table 6).

	FIGO		Age at the diagnosis		Histotype		Risk	
	Sp. index	P value	Sp. index	P value	Sp. index	P value	Sp. index	P value
LHR	0,046	0,61	0,012	0,89	0,049	0,59	-0,20	0,025
KCNH2	-0,228	0,01	0,015	0,87	0,15	0,095	-0,213	0,019
KCNA7	-0,140	0,12	0,044	0,63	0,14	0,118	-0,214	0,018

	Grading		>50% myometrial invasion		Postmenopause		BMI	
	Sp. index	P value	Sp. index	P value	Sp. index	P value	Sp. index	P value
LHR	-0,158	0,082	-0,224	0,014	0,087	0,341	0,146	0,113
KCNH2	-0,172	0,057	-0,192	0,036	0,175	0,053	0,116	0,206
KCNA7	-0,091	0,317	-0,126	0,018	0,179	0,048	0,072	0,435

Table 6: Table representing the associations between LHR, KCNH2 and KCNA7 gene expression and clinical-pathological parameters, calculated with Spearman method. P values associated to the Spearman index (Sp. index).

Subsequently, analyzing the follow-up data, it emerged that ten patients belonging to the case series developed recurrence. Univariate analysis showed a relationship between patient relapse free survival time and the LHR gene expression. Overall, LHR low-expression shows a trend of increased probability of patients recurrence (Kaplan-Meier, Long-rank test, $p=0.17$). Low-expression and high-expression were assigned on the bases of a threshold of 73,78, that represent the median of LHR expression values.

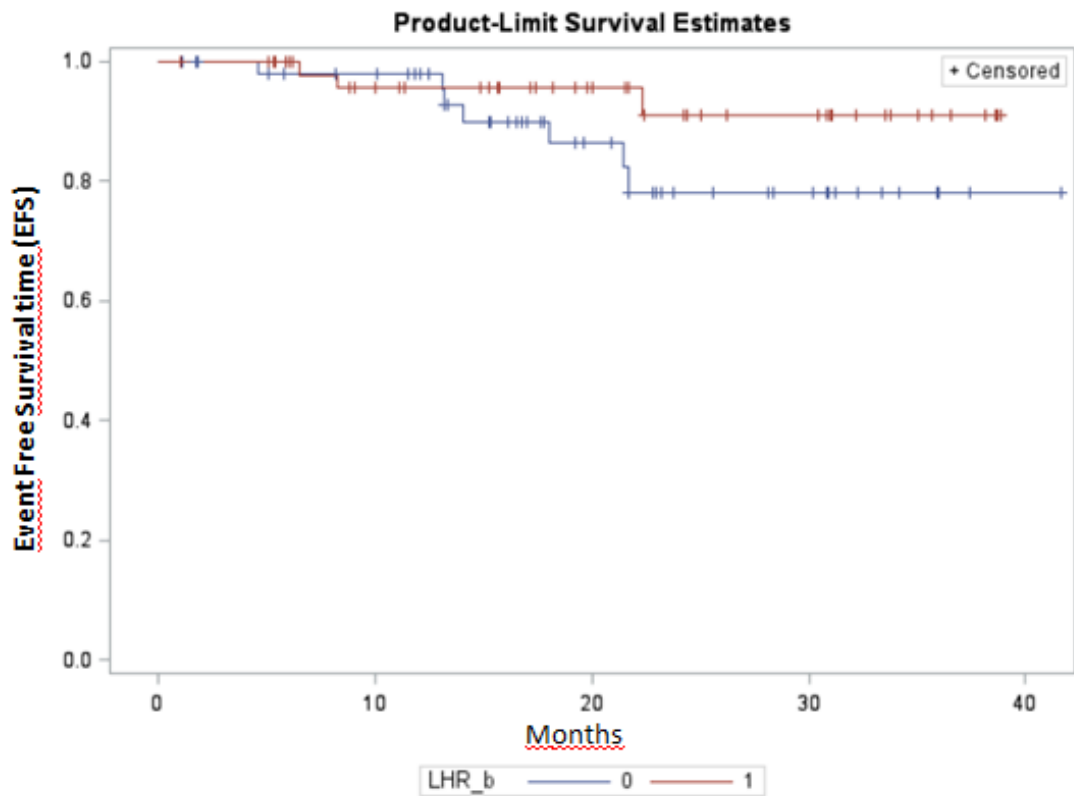


Figure 15: Kaplan-Meier curve of event free survival time (EFS): LHR low-expression shows a trend of increased probability of patients relapse not statistically significant. Long-rank test, $p=0.17$. LHR expression greater than the median=1; LHR expression lesser than the median=0.

We then studied in detail the patients which developed recurrences, it emerged that four out of ten relapsed cases belong to low risk EC. LHR is expressed at high levels (greater than the median value) in two out of four low risk EC patient, while one out of four is expressed slightly below to the median threshold value (table 7). We can notice that two samples (Fi-67 and Fi-156) relapsed earlier than other cases, despite they belong to low risk group. In these two cases LHR expression value is far higher than the median LHR value. Moreover, the patient who developed the relapse earlier (Fi-156), showed an expression of LHR, KCNH2 and KCNA7 genes higher than the median values of each one of the three genes.

Samples	Relapse free survival time	LHR	KCNH2	KCNA7	Risk	Stage	Myometrial invasion
Fi-2	22 months	1	0	0	high	I	>50%
Fi-10	17 months	0	0	0	high	II	>50%
Fi-23	21 months	0	1	1	high	I	>50%
Fi-27	14 months	0	0	0	high	I	>50%
Fi-58	11 months	0	0	0	high	I	>50%
Fi-67	9 months	1	0	1	low	I	<50%
Fi-78	21 months	0	1	0	low	I	<50%
Fi-85	13 months	0	1	1	high	I	>50%
Fi-88	17 months	0	0	1	low	I	<50%
Fi-156	6 months	1	1	1	low	I	<50%

Table 7: table resuming the ten relapsed cases belonging to prospective case series. LHR, KCNH2 and KCNA7 expression values are divided into two groups: 1= the expression value is higher than the median, 0= the expression value is less than the median value.

5. DISCUSSION

Endometrial cancer (EC) is nowadays the most common gynecologic malignancy and the most frequent among infiltrating tumor of the female genital tract. The incidence is rising and five-year survival is worse today than in the past. Although our understanding of the pathophysiology of EC has improved, the results of potential therapeutic options, especially non-surgical treatments, have been disappointing. According to histopathological criteria, EC can be divided into two major types: type I and type II. Type I cancers, which account for 80-90% of sporadic tumors, are usually estrogen-dependent endometrioid adenocarcinomas and are preceded by endometrial hyperplasia. Conversely, type II tumors are a heterogeneous, poorly differentiated group of tumors, usually have late presentation, more aggressive behavior, and are characterized by poor outcome. However, there are women with estrogen-dependent, well-differentiated type I EC relapsing earlier than expected and 1 out of 3 women dying of EC is thought to have early-stage locoregional disease at diagnosis (Jemal A et al 2004; Amant F et al 2005). Our abilities to identify patients at increased risk of relapse or patients more likely to better respond to therapy, are suboptimal. Deciphering the key factors/pathways responsible for the aggressiveness of cancers is mandatory, in order to better stratify EC patients and ensuring the best treatment options. In recent years it was established that LHR is positively involved in EC local invasion and metastatic spread (Pillozzi S et al 2013). In order to identify additional biomarkers that could integrate LHR expression data analysis, we focused on ion channel genes because the proteins encoded by such genes are involved in tumor progression of several different types of cancer. In particular, KCNH2 (hERG1) potassium channel was

described to be up regulated in endometrial cancer (Cherubini, 2000). Thus, a whole-genome expression analysis was previously conducted and we identified a number of ion channels, including KCNH2, whose expression profiles may suggest their involvement in endometrial cancer (Fortunato A PhD thesis). Among these genes we focused on KCNH2 and KCNA7 genes because they seem to be the most dysregulated ion channel genes. For this reason we investigated the LHR, KCNH2 and KCNA7 expression in a prospective cohort of 123 primary EC samples provided by Azienda Ospedaliero-Universitaria, Careggi, Florence. The distributions of LHR, KCNH2 and KCNA7 expression values showed a similar trend for these three genes, however, median expression values are different. Statistical analysis showed significant correlations between the expression level of each one of the three genes. Then, we investigated whether LHR, KCNH2, KCNA7 and clinical-pathological parameters are associated. Significant correlations were found between the high expression of LHR, KCNH2 and KCNA7 and low-risk EC subgroup and between the high expression of the three genes and invasion less than 50% myometrial depth. Moreover, high KCNA7 expression is statistically correlated with postmenopause, while KCNH2 is associated with FIGO stage, so that samples belonging to low FIGO stages show a higher KCNH2 median expression values. Subsequently, we analyzed the follow-up data and it emerged that ten patients developed recurrence. The majority of the relapsed cases belong to high risk EC, with an infiltration level greater than 50% myometrial depth, on the contrary four out of ten relapsed patients belong to low risk EC, stage I and an infiltration level lesser than 50% myometrial depth. We observed that the two cases with the shorter time of relapse belonged to the low risk subgroup and showed a high level of LHR and KCNA7 expression. One of these two cases, which had 6 months of recurrence-time, showed also a high level of KCNH2 expression. Taken together, this data may suggest that these three genes, in particular LHR, may have a role in recurrence-time and in the

developing of a more aggressive phenotype that is not in agreement with type I histopathological classification. In other words, LHR, KCNH2 and KCNA7 could identify patients with low-risk EC characterized by higher probability to relapse.

The study reported in this PhD thesis, is prospective one, therefore the follow-up time is different among patients and the small number of relapse is probably due to the short follow-up period. For these reasons the results are still preliminary and not conclusive and it will be necessary to complete the study analyzing a bigger cohort of patients for a longer period thus expanding both the number of patients enrolled and the follow-up time. Overall, in the field of endometrial cancer these three genes could contribute to better stratify EC patients belonging to the low-risk subgroup.

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